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AGGLUTININ RESPONSES TO S. TYPHI H ANTIGEN IN INTACT AND SUBLETHALLY IRRADIATED MICE FOLLOWING THE INTRAPERITONEAL INJECTION OF HOMOLOGOUS SPLEEN CELL SUSPENSIONS[1]

GORDON O. BAIN

With the technical assistance of MARTIN VAN KESSEL

Abstract

Agglutinin production was depressed in unirradiated mice challenged with *Salmonella typhi* H antigen after intraperitoneal transfer of spleen cells from immunized or non-immunized homologous donors. The inhibition of agglutinin production in the homologous situation and the production of agglutinins by mouse tissues appear to be separate immunological phenomena differing in radiosensitivity. This conclusion is based on the observations that the inhibitory effect was abolished by 500-r but not by 200- or 400-r whole-body irradiation of the recipients before spleen cell transfer, and agglutinin production was depressed more by 400 r plus homologous cell transfer than by the latter procedure alone. Thus, radiation doses of 400 r contributed to depression of antibody production without demonstrably lessening the inhibitory effect attributed to the homologous state.

Introduction

The effects of irradiation on agglutinin production in mice have been studied by Gengozian and Makinodan (1). The studies of Stavitsky (2) and Harris (3) have demonstrated in rats and rabbits that antibody-forming cells stimulated by antigens will produce circulating antibodies after transfer to fresh homologous recipients. If the recipients are exposed to whole body X radiation 1 day before transfer of the antigenically stimulated cells, agglutinins appear in their blood in higher titer than in unirradiated recipients (3). Irradiation in sufficient dosage, depending upon the animal species and the parameters chosen, depresses endogenous antibody production, but has a permissive effect on antibody formation by unirradiated homologous cells injected into irradiated recipients. This observation implies that there is an inhibiting effect of radiosensitive recipient tissues on antibody production by donor cells. On the other hand, the effect of the donor cells on antibody production in the homologous recipient also deserves attention in view of the late lethal results of the transfer of homologous spleen cells to some intact or sublethally irradiated mice. The present report concerns the agglutinin response to a standard test antigen in mice which have received homologous spleen cells.

[1]Manuscript received November 2, 1959.
Contribution from the Department of Pathology, University of Alberta, Edmonton, Alberta. This work was done under Medical Research Grant Number 131, University of Alberta.

Materials and Methods

About seventy-five mice, 6 to 10 months old, descended from AKR and C58 strains each maintained by random intrastrain mating for 3 years, were used. Sera of all animals tested were negative for agglutinins against *Salmonella typhi* H antigen prior to administration of the test antigen, *S. typhi* H (McFarland No. 3).

Donors of spleen cells to groups II, III, IV, and V were injected intraperitoneally with .05 ml of *S. typhi* H suspension 7 days before sacrifice of the animals. Donors to group VI were not immunized. Cells for intraperitoneal injection were teased from the freshly excised spleens with the edge of a fine sterile screen, suspended in Tyrode's solution or saline, and injected immediately. The number of cells injected was estimated from cell counts on aliquots of the cell suspensions and was found to vary from 2 to 10×10^7 cells per recipient.

Certain of the spleen cell recipients (groups III, IV, and V), 24 hours before administration of the test antigen, were exposed to whole-body X radiation in a 20×20 cm partitioned lucite chamber under the following conditions: 200 kv, h.v.l. 1.6 mm Cu, target-object distance 50 cm, dose rate 60 r/minute. Spleen cell transfer was done 1 to 4 hours after irradiation.

Blood samples for agglutinin titration were collected from the tail, allowed to clot, and the serum was separated and frozen pending titration. Samples were taken 48, 72, 96, 125, 144, 168, 192, and 216 hours after injection of the test antigen. Titers were determined in doubling dilutions of serum set up with automatically filling capillary micropipettes. Serum–antigen mixtures were incubated 1 hour at 37° C as sealed hanging drops and read under low power microscopy after 10 and 30 minutes at room temperature.

The experimental groups were arranged as follows: Group I: 12 animals were injected intraperitoneally with the test antigen. Group II: seven mice were injected intraperitoneally with spleen cell suspensions and challenged with the test antigen, administered intraperitoneally, 24 hours later. Group III: six animals were exposed to 500-r whole-body radiation followed by intraperitoneal spleen cell transfer. The test antigen was given 24 hours later. Group IV: eight animals were exposed to 200-r whole-body radiation followed by intraperitoneal transfer of spleen cells. Twenty-four hours later, the test antigen was injected intraperitoneally. Group V: six mice were exposed to 400-r whole-body radiation followed by intraperitoneal injection of spleen cell suspensions. The test antigen was injected 24 hours later. Group VI: 12 mice were injected intraperitoneally with spleen cells from non-immunized donors, and with the test antigen 24 hours later.

Results

In response to intraperitoneal injection of *S. typhi* H antigen mice of our colony produce agglutinin titer curves like that in Fig. 1. After an induction period of 40 to 50 hours the titer rises rapidly reaching a peak at the average time of 137 hours (S.D. 15) after antigen injection. The titer then falls and

subsequently rises more gradually. In the present study the term peak titer refers to the peak of the initial rise.

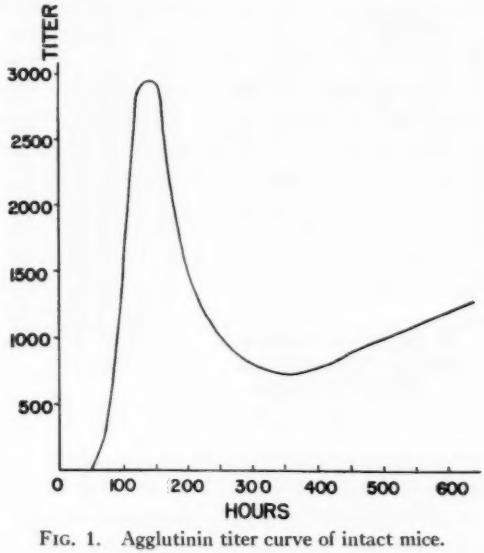

FIG. 1. Agglutinin titer curve of intact mice.

The experimental groups with their mean log (base 10) peak titers and standard deviations are shown in Table I. Peak titers used to calculate the means were actually observed values rather than estimations from smoothed curves. Mean log (base 10) agglutinin titers and mean log (base 10) peak titers are shown in Fig. 2.

TABLE I

Mean peak agglutinin titers of mice

Group	I	II	III	IV	V	VI
Treatment	AG	HSC–AG	500r–HSC–AG	200r–HSC–AG	400r–HSC–AG	HSC*–AG
Mean peak $\log_{10}$ titer	3.34	2.63	3.61	2.58	1.90	3.03
Standard deviation	0.30	0.62	0.69	0.71	0.32	0.35
N	12	7	6	8	6	12

NOTE: AG: *Salmonella typhi* H administered intraperitoneally.
HSC: Homologous spleen cells from immunized donors injected intraperitoneally.
HSC*: Homologous spleen cells from non-immunized donors injected intraperitoneally.
N: Number of mice.

Comparison of groups I and II shows that, in mice which had received intraperitoneal injection of homologous spleen cells (hereafter called HSC mice) from immunized donors, the production of circulating antibody in response to injection of the test antigen was significantly depressed as reflected by depression of the mean peak log titer (S.E. of difference = 0.25) and delay of the time of maximum increment of agglutinin titer (S.E. of difference = 6.5,

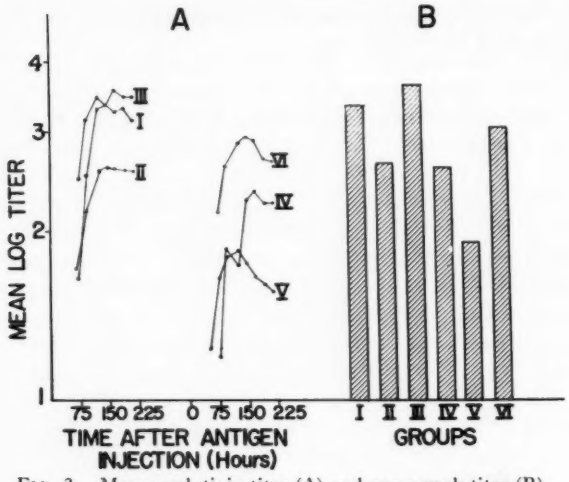

FIG. 2. Mean agglutinin titer (A) and mean peak titer (B).

means 94 and 117 hours and S.D. 11 and 15 respectively). The depressant effect on peak titer was abolished by prior irradiation in group III at a dose level of 500 r (S.E. of difference = 0.12). Radiation doses of 200 r and 400 r (groups IV and V) did not mitigate the depressant effect. No significant difference between the mean peak titers of HSC mice preirradiated at a dose of 200 r and unirradiated HSC mice was demonstrated, but those which had received 400 r reached a mean peak titer significantly lower than unirradiated and 200 r groups (S.E. of difference .27 and .28 respectively). This suggests that a dose of 400 r augmented depression of the antibody response in the presence of homologous cells. The mean peak titer attained by recipients preirradiated with 500 r did not differ significantly from that of untreated mice exposed to the test antigen, but was significantly higher than that of all other groups of HSC mice. The mean peak titer of unirradiated recipients of spleen cells from the non-immunized donors (group VI) was significantly lower than that of mice receiving no homologous tissue (group I), (S.E. of difference .14). This suggests that the depression of antibody production observed in HSC mice which had received spleen cells from immunized donors is not satisfactorily accounted for by the transfer of antibody with the spleen cell injection.

Only two animals, both recipients of homologous spleen cells, died during the 9-day observation period concerned in the present report. One had been irradiated; the other had not. Both had received spleen cells from the same donor. Several additional deaths occurred later among spleen cell recipients. All of these animals were subjected to autopsy examination and no evidence of leukaemia was found.

Discussion

The data of Makinodan and Gengozian (4, 5) concerning the agglutinin response of mice to an intraperitoneal injection of sheep and rat red blood

cells show an induction period and primary rise in titer similar to that in the present study. Their results do not, however, show the early agglutinin peak which was a consistent finding in our animals in response to *S. typhi* H antigen. The absence of such an early peak in their data may be ascribable to the different antigen or to the method of study. The nature of the agglutinin responses of mice to these antigens renders differentiation of primary and secondary responses very difficult. It may even be questioned whether a true primary response occurs, but this raises a matter of definitions which is not strictly pertinent to the purposes of the present report. It may simply be stated that agglutinin titer curves of mice exposed for the first time to *S. typhi* H antigen did not differ from those of mice exposed to the antigen after intraperitoneal injection of spleen cells previously exposed to the same antigen in a manner which would permit their characterization as primary and secondary responses respectively.

The agglutinin responses in mice which have been injected with spleen cells from other mice presumably represent the sums of agglutinin elaboration by the donor cells and the recipient's own immunological system. In the present experiment no attempt was made to distinguish between donor cell and recipient antibody production. The titers observed are regarded as reflecting agglutinin formation in a homologous complex. Under these circumstances the levels of antibody production may be affected by influences exerted by one cell population upon the other or by both cell populations upon one another. Whole body irradiation of the recipient animal is known to alter its immunological responsiveness but it also alters the environment into which the donor cells are introduced.

Antibody production in response to the bacterial test antigen was depressed in HSC mice. This effect cannot be ascribed to the overwhelming of immunological mechanisms by homologous antigen, since the depressant influence was abolished if the recipient had been previously exposed to 500 r whole body X radiation.

The inhibition of antibody production in HSC mice appears to be a specific influence resistant to radiation doses of 200 and 400 r but sensitive to 500 r whole-body irradiation of the recipient before spleen cell transfer. Antibody production was more depressed by 400 r plus homologous spleen cell transfer (mean peak titer 1.90) than by homologous cell transfer alone (mean peak titer 2.63). Inhibition of antibody production was not abolished by irradiation of the recipient with 400 r but was abolished by 500 r (mean peak titer 3.61). These observations suggest two separate functions of the recipient tissues. If antibody production in response to the test antigen and inhibition of antibody production by a homologous environment were similar immunological functions, it would be expected, in accordance with the hypothesis of Makinodan and Gengozian, that the latter, a reaction to closely related antigens, would be abolished at a lower radiation dose than the former, a response to an unrelated antigen (4).

In their study of the antibody response of mice after whole-body irradiation, Makinodan and Gengozian (5) found that doses of 100 r, 300 r, and 500 r had

little effect on the mean peak agglutinin titer to sheep red cells injected 24 hours after irradiation. This may suggest that in the present experiment depression of mean peak titer in the 200 r group is attributable mainly to the homologous situation. In the 400 r group, irradiation and spleen cell transfer probably exerted additive depressant effects. Although the results include no data directly pertinent to the question, it seems likely that, in group III, preirradiation of the recipients with 500 r was sufficient to permit transplantation and virtually uninhibited antibody synthesis by homologous donor cells that had been antigenically stimulated.

The observation of late deaths among our recipients of spleen cells and the report of Cole and Ellis (6) of a mortality of 89% among non-irradiated mice injected intraperitoneally with homologous spleen cells indicate that the radiosensitive depression of antibody formation is only one facet of a complex situation. The degeneration of antibody-forming tissues in F_1 hybrid mice injected with parental strain spleen cells, recently reported by Nowell and Cole (7), may be a morphological correlate of the depression of antibody production observed in the present study.

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STUDIES OF HYDRANGENOL IN HYDRANGEA MACROPHYLLA SER.

I. ISOLATION, IDENTIFICATION, AND BIOSYNTHESIS FROM C^{14}-LABELLED COMPOUNDS[1]

RAGAI K. IBRAHIM AND G. H. N. TOWERS

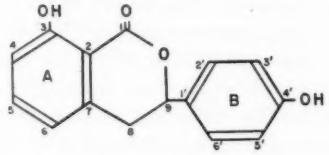
Abstract

Hydrangenol glucoside was isolated and identified from the flowers of *Hydrangea macrophylla* Ser. The free isocoumarin, hydrangenol, was isolated and identified from acid hydrolyzates of flower, leaf, and root extracts of the same species. Roots contained the largest amounts.

Cinnamic acid-2-C^{14} and phenylalanine-U-C^{14} were relatively good precursors of hydrangenol-C^{14}, whereas tyrosine-U-C^{14} was not. Similarly, hydrangenol-C^{14} was readily synthesized by roots from acetate-1-C^{14} and acetate-2-C^{14}. This preliminary study suggests that both acetate and phenylpropanoid compounds may be involved in hydrangenol synthesis.

Introduction

Hydrangenol is an isocoumarin with the following structure:



It was first isolated by Asahina and Mujake (1) from sepals of *Hydrangea hortensia* Dipp. and its identity confirmed by synthesis from 3-methoxyphthalic anhydride and *p*-methoxyphenylacetic acid (2). Further investigations by Ueno (3, 4) resulted in the isolation from the same source of hydrangenol glucoside. In more recent studies of the phenolic constituents of the sepals of *Hydrangea macrophylla* (5, 6), no reference is made to the presence of hydrangenol or of its glucoside in this species.

Considerable interest is being shown in the biosynthesis of aromatic compounds in higher plants. Evidence for the formation of benzene rings from acetate or from shikimic acid via phenylpropanoid compounds has been obtained for a number of phenolic compounds. It has been shown that the A ring of an anthocyanidin (7, 8), a flavonol (9, 10), and a dihydrochalcone (11) are formed from acetate in accordance with the hypothesis of Birch and Donovan (12); while the B ring and the C_3 side chain are more readily formed from phenylpropanoid compounds such as phenylalanine. The biogenesis of coumarin, a C_6—C_3 lactone (13, 14) has also been shown to proceed via phenylpropanoid compounds.

The carbon skeleton of hydrangenol resembles a stilbene in structure with one more carbon on ring A forming a lactone. A number of stilbenes occur

[1]Manuscript received February 10, 1960.
Contribution from the Botany Department, McGill University, Montreal, Que.

naturally in plants and Robinson (15) has suggested that they may be formed exclusively from acetate units, i.e. both rings being derived from acetate. He has given examples of stilbenes in which the orientation of the hydroxyl groups agrees with this proposed scheme of biogenesis. On the other hand, from a biogenetic standpoint, hydrangenol may not be closely related to the stilbenes and it is possible that the B ring and carbons 7, 8, and 9 of hydrangenol are formed from a phenylpropanoid precursor, while carbons 1–6 are derived from acetate. This would be in agreement with the hypothesis of Birch and Donovan.

The novel structure of hydrangenol prompted us to study its biogenesis. The work reported in this paper is mainly concerned with the methods of isolation and identification of hydrangenol and its glucoside. A preliminary study of the biogenesis with the aid of C^{14}-labelled compounds considered to be likely precursors is also included.

Experimental and Results

Isolation of Hydrangenol Glucoside

Four hundred and forty grams of fresh flowers of *Hydrangea macrophylla* Ser., obtained from the Montreal Botanical Garden, were extracted with boiling 85% ethanol under reflux for 12 hours with two changes of ethanol. The combined ethanolic extracts (4.5 liters) were reduced in volume by distillation under reduced pressure in a cyclone evaporator to a thick syrup, which was taken to dryness under a jet of filtered air at room temperature. The brown residue (18 g) was dissolved in 300 ml of hot water, filtered hot through a bed of Celite, and continuously extracted with ethyl acetate for 48 hours. The ethyl acetate extract was reduced in volume and kept under refrigeration over night when a gummy precipitate came down. The precipitate was filtered off and dissolved in absolute ethanol. The ethanolic extract was filtered, evaporated to dryness, and the residue dissolved in water, acidified with HCl, and continuously extracted with ether for 16 hours. The aqueous extract was reduced in volume by means of an air jet and kept in a refrigerator where the glucoside came down as a pale cream-colored powder (m.p. 184.5–185.5° C uncorr.). It was purified by re-extraction of an aqueous solution with ethyl acetate. The solvent was removed and the residue was recrystallized twice from hot water to give 261 mg of fine white needles which melted at 190–190.5° C uncorr. (reported 172° C (3) or 192° C (4)).

Identification of Hydrangenol Glucoside

Hydrangenol glucoside has a faint blue fluorescence in ultraviolet light. Its absorption maximum determined in 85% ethanol using a model DU Beckman spectrophotometer was found to be at 315 mμ (ϵ 1790) as shown in Fig. 1. In alkaline ethanol solution its absorption maximum was shifted to 357 mμ. Elemental analysis of the glucoside gave C, 60.23, 59.95; H, 5.50, 5.35; calculated C, 60.5, H, 5.27.

The isolated hydrangenol glucoside was chromatographically pure. On two-directional descending paper chromatograms using (80:20) phenol:water in the first direction and (4:1:5) butanol:acetic acid:water in the second, it had R_f values of 0.60 and 0.47 respectively. It reacted with 1% ferric chloride

solution giving a light red-brown color. It gave a pink color with diazotized *p*-nitroaniline oversprayed with sodium hydroxide and a yellow color with alkaline diazotized sulphanilic acid.

Hydrolysis of hydrangenol glucoside by refluxing with 5% HCl on a steam bath for an hour gave glucose and hydrangenol. The identity of the products was proved by cochromatography with authentic samples.

Isolation of Hydrangenol from Flowers

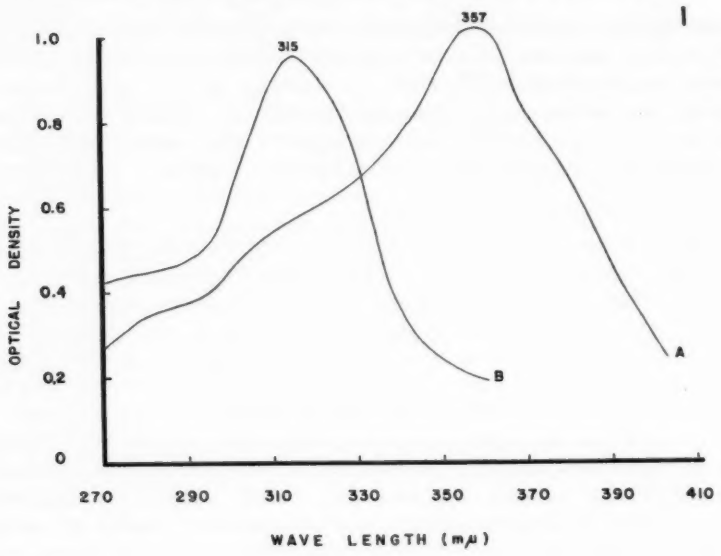
Air-dried flowers of *H. macrophylla* (1.5 kg) were continuously extracted with 85% ethanol in large Soxhlets for 24 hours with two changes of ethanol. The combined ethanol extracts (10 liters) were reduced under vacuum to a thick syrup which was suspended in two volumes of water and hydrolyzed with 10% HCl for an hour on a steam bath when a tar-like residue separated. The acid hydrolyzate was decanted and extracted with ether in a continuous extractor for 12 hours. The tar-like residue was exhaustively extracted with ether on the steam bath and the insoluble residue was discarded. The combined ether extracts were washed in a separatory funnel with 5% Na_2CO_3 solution until the alkaline extracts were colorless. This removed the phenolic acids. Since hydrangenol is slightly soluble in carbonate solution, the alkaline extracts were re-extracted with ether. The combined ether extracts were washed twice with water and extracted with 10% NaOH solution in a separatory funnel. On acidification of the alkaline extract with concentrated HCl, hydrangenol came down as a grey-green precipitate. It was allowed to stand at room temperature, filtered, and then dried *in vacuo* (4.5 g). It was recrystallized twice from hot 20% ethanol to give 2.0 g of white needles, m.p. 180–181° C uncorr. (reported 181–182° C (1)).

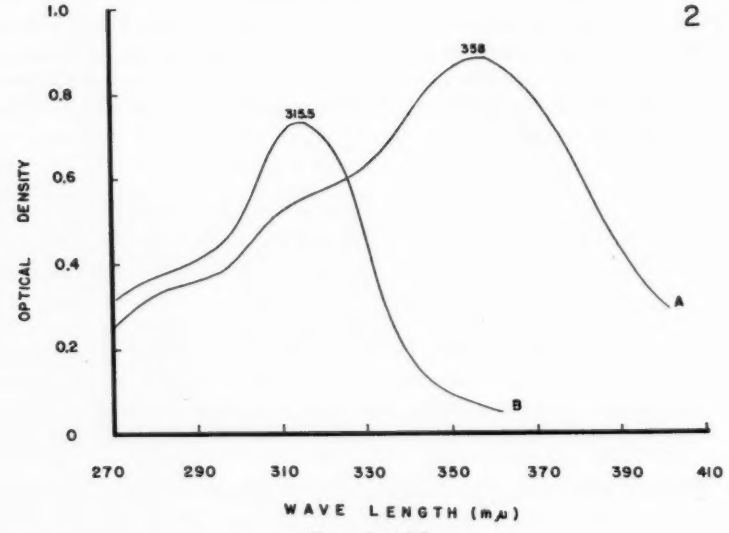
Identification of Hydrangenol

Hydrangenol has a strong blue fluorescence in ultraviolet light. The absorption maximum was found to be 315.5 mμ (ϵ 4310) in 85% ethanol and 358 mμ in alkaline ethanol solution as shown in Fig. 2. Elemental analysis of hydrangenol gave C, 70.69, 70.93; H, 4.89, 4.79; calculated C, 70.3, H, 4.6.

Hydrangenol was similar in its chromatographic behavior to its glucoside. In (80:20) phenol:water, followed by (4:1:5) butanol:acetic acid:water, it had R_f values of 0.77 and 0.80 respectively; and an R_f of 0.72 in (6:7:3) benzene:acetic acid:water. It gave a violet color with 1% ferric chloride, a pink color with diazotized *p*-nitroaniline, and an orange color with diazotized sulphanilic acid.

The diacetyl derivative of hydrangenol was prepared by allowing a mixture of hydrangenol (0.15 g), acetic anhydride (0.7 ml), and dry pyridine (1.5 ml) to stand under nitrogen in a refrigerator for 24 hours. The mixture was poured into ice-water and the crystalline compound was filtered, dried *in vacuo*, and recrystallized from boiling absolute ethanol to give glistening white plates (yield 91%) which melted at 182–183° C uncorr. (reported 181–182° C (1)). Elemental analysis of the diacetyl hydrangenol gave C, 67.36, 67.62; H, 5.04, 5.27; calculated C, 67.06, H, 4.7.





FIGS. 1 and 2.

Degradation of Hydrangenol

Hydrangenol was fused with sodium hydroxide pellets in a stainless steel bomb with a steel cap for 20 minutes in a Wood's metal bath maintained at 300–350° C. The hydrolyzed mass was allowed to cool, dissolved in a small amount of water, and carefully acidified with concentrated HCl. The acidified

hydrolyzate was continuously extracted with ether for 6 hours, taken into 5% sodium carbonate, and the latter was re-extracted with ether after being acidified. Chromatography of the final ether extract in (6:7:3) benzene:acetic acid:water in the first direction, followed by (10:1:200) sodium formate:formic acid:water in the second, gave three compounds the R_f values and color reactions of which are shown in Table I.

TABLE I

R_f values and color reactions of the degradation products of hydrangenol

Compound	R_f values		Color reactions	
	Solvent 1*	Solvent 2†	Diazotized *p*-nitroaniline/NaOH	Diazotized sulphanilic acid/NaOH
A	0.25	0.75	Pink	Yellow
B	0.83	0.33	Deep-purple	Orange-yellow
C	0.50	0.10	Light-blue	Buff

*Benzene:acetic acid:water (6:7:3).
†Sodium formate: formic acid:water (10:1:200).

Compound A was identified as *p*-hydroxybenzoic acid by cochromatography with an authentic sample. Compound B was isolated in a small quantity by chromatographic separation following the above-mentioned procedure. It was recrystallized twice from hot water, giving colorless needles which softened at 145° C and decomposed at 167–168° C, uncorrected (reported m.p. of 2,6-cresotic acid 168° C (16)). Elemental analysis gave C, 62.73, 62.84; H, 5.63, 5.56; calculated for 2,6-cresotic (2-hydroxy-6-methylbenzoic) acid C, 63.1, H, 5.3. Both *p*-hydroxybenzoic and 2,6-cresotic acids were reported to be fusion products of hydrangenol (1). Compound C appeared to be an intermediate product in the degradation procedure. It occurred in very small amounts on paper chromatograms and was not identified.

Since the yield of 2,6-cresotic acid obtained from the alkali fusion of hydrangenol was relatively small (5% of theoretical), further attempts were made to degrade hydrangenol using different concentrations of alkali and different hydrolysis periods. None of these treatments gave better yields of *p*-hydroxybenzoic or 2,6-cresotic acids. In most cases relatively large amounts of unchanged hydrangenol were obtained. In some others, at least 10 unidentified phenolic compounds were detected on paper chromatograms.

Occurrence of Hydrangenol Glucoside in Different Organs of H. macrophylla

From a number of analyses, the average yields of hydrangenol after acid hydrolysis per 100 g fresh weight were found to be: 100 mg for flowers, 40 mg for leaves, and 250 mg for roots. The hydrangenol isolated from the roots was comparatively pure and required only one recrystallization to give a product that had a correct melting point and was chromatographically pure. No free hydrangenol was detected in any part of the plant, but it was isolated after acid hydrolysis of the plant extracts.

Leaves, roots, and bark of three other species of *Hydrangea* (*H. bretschneideri* Dipp., *H. arborescens* Linn., and *H. cinerea* Small) were examined, but none of them was found to contain hydrangenol.

Biosynthesis of Hydrangenol from C^{14}-labelled Compounds

Since the roots of *H. macrophylla* contained appreciable amounts of hydrangenol glucoside and since flowers were not available at the time, roots were used as experimental material.

In one experiment, 2-month-old rooted cuttings, each having about ten leaves were used. The fresh weights of the roots of these cuttings ranged from 12.4–17 g. The soil was removed from the roots, which were thoroughly washed with tap water and blotted with filter paper. The roots were placed in small beakers, each containing a C^{14}-labelled compound in 3 ml of distilled water. Sodium cinnamate-2-C^{14}, L-phenylalanine-U-C^{14}, L-tyrosine-U-C^{14}, and sodium acetate-2-C^{14}, all purchased from Merck Radiochemical Labs. Montreal, were used. The plants were left to stand under a bank of fluorescent lights for 48 hours during which time the radioactive solutions were absorbed and water was added as required. At the end of this period, the root systems were cut off, thoroughly washed with water, and dried with filter paper. They were chopped and rapidly plunged into boiling 85% ethanol and worked up for the isolation of hydrangenol following the procedure mentioned before. The hydrangenol content of the roots ranged from 0.8–1.7 mg/g fresh weight. The isolated hydrangenol was recrystallized and its melting point determined. An accurately weighed amount was dissolved in 1 ml of 95% ethanol and duplicate samples were plated for radioactivity determination. Samples were plated on aluminum planchettes to contain less than 200 $\mu g/cm^2$ and therefore counted at infinite thinness. As a result no correction for self-absorption was made. All counts were taken to a mean error of 2% or better. The equipment used was a Berkeley model 2001 decimal scaler and a type IWAAA Geiger–Muller tube (end window thickness 1.25 mg/cm^2). The relative efficiency of the instrument was determined to be 8.5%.

Since the specific activities of the hydrangenol isolated from the first experiment were considered low, a second experiment was carried out on 1.5-g

TABLE II

Synthesis of hydrangenol from C^{14}-labelled compounds in *Hydrangea* roots

Compound	Compound administered		Hydrangenol		
	Amount (mmole)	Specific activity (μc/mmole)	Amount isolated (mmole)	Specific activity (μc/mmole)	Dilution*
	First experiment				
Sod. cinnamate-2-C^{14}	0.0062	960	0.057	0.72	1,330
Sod. acetate-2-C^{14}	0.0009	1,150	0.043	0.85	4,050†
L-Phenylalanine-U-C^{14}	0.0009	13,200	0.130	1.14	11,600
L-Tyrosine-U-C^{14}	0.0005	12,500	0.058	0.039	320,500
	Second experiment				
Sod. cinnamate-2-C^{14}	0.0071	989	0.0099	2.10	470
L-Phenylalanine-U-C^{14}	0.0007	10,500	0.015	3.81	2,760
Sod. acetate-2-C^{14}	0.0012	1,150	0.011	0.56	6,150†
Sod. acetate-1-C^{14}	0.0020	1,550	0.029	0.73	6,390†

*Specific activity of the labelled compound (μc/mmole) by specific activity of hydrangenol (μc/mmole). In calculating dilution values, no corrections have been made for the amounts of hydrangenol isolated.

†The dilution values of acetate feedings in both experiments were calculated on the basis of three molecules of acetate being incorporated into one molecule of hydrangenol.

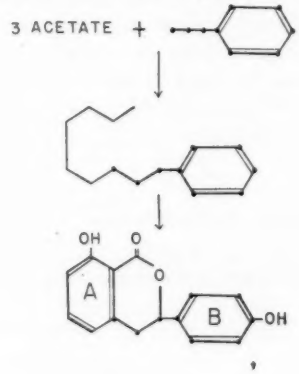
samples of root cuttings. In this experiment, sodium cinnamate-2-C^{14}, L-phenylalanine-U-C^{14}, sodium acetate-1-C^{14}, and sodium acetate-2-C^{14} were used. The amounts of hydrangenol recovered from these roots ranged from 2 to 5 mg/g fresh weight. The results of both experiments were given in Table II.

Discussion

From various analyses involving the isolation of hydrangenol from different organs, it is almost certain that this compound occurs only in bound form as the glucoside and not as the free phenol. Furthermore, the discovery that the roots contain hydrangenol glucoside in amount 2–3 times as great as that of flowers is very helpful in biosynthetic studies. The hydrangenol content of flowers has not been determined at different stages of flowering so as to decide whether the flower or the root is the more suitable for studying hydrangenol synthesis. The administration of labelled precursors to young inflorescences may be valuable.

From the results of both sets of feeding experiments (Table II), it appears that cinnamic acid is a relatively good precursor of hydrangenol compared to other compounds used. L-Phenylalanine-C^{14} was also shown to be incorporated, although to a lesser degree in the first experiment than in the second (Table II), which may be due to the much larger amount of hydrangenol isolated in the first feeding. If these C_6–C_3 compounds are incorporated intact into hydrangenol, the activity should be in the B ring and carbons 7, 8, and 9 from phenylalanine-U-C^{14} feeding and in C 7 from cinnamate-2-C^{14} feeding. Another phenylpropanoid compound, L-tyrosine, in spite of its close structural relationship to phenylalanine, was relatively ineffective as a precursor of hydrangenol. Underhill, Watkin, and Neish (10) showed that tyrosine was a very poor precursor in the biosynthesis of quercetin. Similarly, Hutchinson *et al.* (11) reported that tyrosine was very poorly incorporated into phloridzin in *Malus* tissues, unlike phenylalanine, which was a good precursor. They found that tyrosine was readily metabolized to amino acids and even to sugars.

Acetate-1-C^{14} and acetate-2-C^{14} were also shown to be equally effective in forming hydrangenol-C^{14}. The incorporation of the label of both cinnamic acid and phenylalanine on the one hand, and of acetate on the other, suggests that hydrangenol may be formed in the following way:



Proof of the above mentioned pathway must await the results of further studies involving administration of specifically labelled compounds and degradation of hydrangenol. The low specific activities of hydrangenol obtained in the experiments reported herein precluded degradation studies. Further work along these lines is in progress in our laboratory.

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FATTY ACID, CHOLESTEROL, AND ACETOACETATE BIOSYNTHESIS IN LIVER HOMOGENATES FROM NORMAL AND STARVED GUINEA PIGS[1]

FRANK SAUER

Abstract

Fatty acid, cholesterol, and acetoacetate biosynthesis was studied with liver homogenates from normal and starved guinea pigs. Starvation suppressed the incorporation of acetate into fatty acids and cholesterol but not into acetoacetate. The in vitro biosynthesis of cholesterol and fatty acids was not restored by the addition of either G-6-P or isocitrate in combination with TPN, nor was it restored by orally dosing the starved animals with dextrose. The addition of isocitrate to normal homogenates depressed cholesterogenesis and stimulated lipogenesis.

Introduction

The effects of the physiological state of an animal on the in vitro synthesis of fatty acids, cholesterol, and acetoacetate have been extensively studied. Starvation greatly decreases in vitro biosynthesis of fatty acids and cholesterol (1–6). In vitro lipogenesis is also decreased in alloxan diabetic rats (7–10). Langdon (11) suggested that decreased lipogenesis was the result of a decreased availability of TPNH. Studies by Siperstein and Fagan (12) indicate that cholesterol and fatty acid synthesis in liver homogenates from diabetic rats can be fully restored by the addition of a TPNH-generating system.

On the other hand the in vitro incorporation of labelled acetate into acetoacetate is not depressed by starvation, and acetoacetate production by either liver slices or homogenates generally increases with starvation (6, 13, 14). In these studies the effects of starvation on the synthesis of fatty acids, cholesterol, and acetoacetate were investigated and attempts were made to restore biosynthesis in liver homogenates from starved animals. It is hoped that a study of biosynthetic pathways and acetoacetate formation in the starvation state will ultimately yield information relevant to the problem of acetonemia in ruminants.

Materials and Methods

Female guinea pigs weighing approximately 300 g were raised on a commercial guinea pig diet that contained 24% protein, 4% fat, and 12% fiber. Animals were killed by decapitation and the liver perfused with 20 ml of ice-cold 0.25 *M* sucrose. The perfused liver was homogenized in 2.5 parts phosphate buffer (pH 7.4) that was composed of 0.044 *M* potassium phosphate, 0.03 *M* nicotinamide, 0.007 *M* magnesium chloride, and 0.126 *M* sucrose as described by Bucher (15). Glutathione did not stimulate synthesis and was omitted from the buffer.

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[2]The following abbreviations are used: G-6-P, glucose-6-phosphate; TPN, triphosphopyridine nucleotide; TPNH, reduced triphosphopyridine nucleotide; DPN, diphosphopyridine nucleotide.

The homogenate was centrifuged at 700 × *g* for 5 minutes and 2 ml of supernatant was added to Warburg flasks for a 90-minute incubation period at 37° in an atmosphere of O_2.

Sterol was isolated as the digitonide and counted as described by Migicovsky (16). After the non-saponifiable fraction was removed, the alkaline digest was acidified and extracted several times with petroleum ether. The petroleum ether fraction was washed, dried over anhydrous sodium sulphate, and evaporated under N_2. The fatty acids were redissolved in hot acetone and brought to volume, and measured aliquots were plated infinitely thin on aluminum planchets for counting.

Acetoacetate was decarboxylated with aniline citrate at reduced temperature (17) and the liberated CO_2 counted as barium carbonate. No correction was made for the decarboxylation of oxaloacetic acid since this probably does not introduce a sizeable error (18). Although it may be assumed that the radioactivity in acetoacetate is equally distributed in the carboxyl and carbonyl carbon atoms, the data reported here represent only the activity present in the carboxyl carbon.

Total acetoacetate was determined colorimetrically with *p*-nitroaniline by the method of Walker (19) and the modifications suggested by Kalnitsky *et al.* (20). By subtracting the acetoacetate present before incubation, the net acetoacetate produced by the homogenate during incubation could be determined.

The nitrogen content of the homogenates was determined by micro-Kjeldahl digestion and nesslerization. All values were corrected to a constant nitrogen content in the homogenates.

In the studies summarized in Table IV female guinea pigs weighing 333 g were given four oral doses of 0.5 g sodium citrate in 5 ml water at 6-hour intervals. Animals in the control group were dosed 4 times with 5 ml water. One hour after the last dosing the animals were injected intraperitoneally with sodium acetate-1-C^{14} (230 × 10^6 c.p.m.) and killed 3 hours later. Isolation of fatty acids and cholesterol was carried out according to the method of Van Bruggen *et al.* (21) except that cholesterol was isolated and counted as the digitonide.

Results and Discussion

Liver homogenates prepared from normal guinea pigs incorporate labelled acetate into cholesterol and fatty acids at rates comparable to those observed with rat liver homogenates (Table I) (22). Normal homogenates vary considerably in their ability to incorporate acetate into cholesterol, a finding also observed by others (4, 23).

Adding G-6-P alone, and in combination with TPN, to normal homogenates (Table II) reduces the incorporation of acetate into cholesterol and acetoacetate and slightly increases incorporation into fatty acids. This is at variance with results of Siperstein and Fagan (23), who noted that incorporation of acetate into cholesterol and fatty acids was greatly increased when G-6-P and TPN were added to normal rat liver homogenates. However, the mμmole of acetate

TABLE I

Biosynthesis of cholesterol, fatty acids, and acetoacetic acid in liver homogenates from normal guinea pigs

mμmoles labelled acetate incorporated		Net production of acetoacetic acid (μg)
Cholesterol	Fatty acids	
34.5	3.1	96.8
2.4	1.3	51.9
8.1	1.5	65.3
5.1	1.7	37.9
7.3	3.4	71.5
7.5	2.0	107.0

NOTE: One part liver was homogenized in 2.5 parts buffer containing: potassium phosphate, 0.044 *M*; nicotinamide, 0.028 *M*; $MgCl_2$, 0.007 *M*; sucrose 0.126 *M*. Final vol. 2.4 ml, pH 7.4. Additions: 5 μmoles DPN, 2 μmoles ATP, 1.32 μmoles acetate-1-C^{14}. Incubation 90 minutes; gas phase O_2.

TABLE II

Biosynthesis of cholesterol, fatty acids, and acetoacetic acid by liver homogenates from normal guinea pigs showing effect of added substrate and cofactor

Animal	Addition to incubation mixture	mμmoles acetate-1-C^{14} incorp. into:			μg aceto-acetic acid produced
		Cholesterol	Fatty acids	Acetoacetic acid	
1	Control	1.4	.8	26.6	64.0
	G-6-P (30 μmoles)	1.2	1.7	17.5	65.0
	G-6-P (30 μmoles) + TPN (4 μmoles)	.5	2.4	16.2	49.1
	TPN (4 μmoles)	.9	1.1	27.2	47.1
2	Control	6.4	8.0	76.5	130.4
	G-6-P (30 μmoles)	4.8	9.6	62.8	126.8
	G-6-P (30 μmoles) + TPN (4 μmoles)	3.8	10.4	51.6	75.5
	TPN (4 μmoles)	5.7	8.1	—	—
3	Control	2.5	1.5	106.0	136.0
	G-6-P (30 μmoles)	1.9	2.7	—	107.6
	G-6-P (30 μmoles) + TPN (4 μmoles)	1.5	3.2	81.0	78.5
	TPN (4 μmoles)	1.6	1.7	91.0	72.5

NOTE: Incubation procedure as in Table I; 5 μmoles DPN, 2 μmoles ATP, 1.32 μmoles acetate-1-C^{14} added to all samples.

incorporated into cholesterol in the experiments reported here were similar in magnitude to those found by Siperstein and Fagan following additions of both DPN and TPN.

When G-6-P was replaced with isocitrate (Table III) there was a greater reduction in cholesterol biosynthesis and a marked increase in lipogenesis. This isocitrate effect was only slightly enhanced by the addition of TPN. If this increased lipogenesis is to be explained on the basis of TPNH production via isocitric dehydrogenase it follows that normal guinea pig liver homogenates contain sufficient amounts of this cofactor for optimum lipogenesis.

Dituri *et al.* (24) observed the greatest stimulation in lipogenesis when particle-free systems of pigeon and rat liver were incubated in the presence of 0.01 *M* citrate, and that citrate could not be replaced by G-6-P, TPN, and

TABLE III

Biosynthesis of cholesterol, fatty acids, and acetoacetic acid by liver homogenates from normal guinea pigs showing effect of added substrate and cofactor

Animal	Addition to incubation mixture	mμmoles acetate-1-C^{14} incorporated			μg aceto-acetic acid produced
		Cholesterol	Fatty acids	Acetoacetic acid	
4	Control	30.2	3.7	53.4	55.9
	DL-Isocitrate (21 μmoles)	11.6	39.0	50.6	89.6
	DL-Isocitrate (21 μmoles) + TPN (4 μmoles)	8.9	37.7	33.5	
5	Control	3.4	9.7	92.7	139.0
	DL-Isocitrate (21 μmoles)	.4	80.1	33.8	62.5
	DL-Isocitrate (21 μmoles) + TPN (4 μmoles)	.2	89.6	29.9	34.3
6	Control	4.6	2.2	95.7	42.5
	DL-Isocitrate (30 μmoles) + TPN (4 μmoles)	.7	12.6	55.2	44.5

NOTE: Incubation procedure as in Table I; 5 μmoles DPN, 2 μmoles ATP, 1.32 μmoles acetate-1-C^{14} added to all samples.

G-6-P dehydrogenase. The addition of isocitrate and TPN lowers the incorporation of labelled acetate into acetoacetate. This has been explained (10) on the basis that a high TPNH–TPN ratio would divert acetoacetyl CoA and B-hydroxybutyryl CoA in the direction of butyryl CoA via the reduction of crotonyl CoA, which Langdon (25) has shown to be TPN-dependent.

On the basis of these considerations the short-term effects of sodium citrate were tested in vivo. The results (Table IV) differed from those observed in vitro with isocitrate in that the specific activity of the liver fatty acids was

TABLE IV

The in vivo effects of dosing guinea pigs orally with sodium citrate

Group	No. of animals	c.p.m. per mg cholesterol digitonide	c.p.m. per mg fatty acids
Control	5	261 ± 43.9*	745 ± 125.9*
Experimental	7	160 ± 39.5	409 ± 46.8
		$P > .05$	$P < .05$

*Standard error of mean.

decreased ($P < .05$) in the citrate fed group, while the decrease in the specific activity of the cholesterol was not significant ($P > .05$). These results indicate that although citrate influences in vivo biosynthesis, the effects are different from those observed in vitro. This illustrates the difficulties encountered in comparing results obtained with the intact animal to those observed in isolated systems.

When guinea pigs were starved for a period of 25 hours there was a marked reduction in the in vitro incorporation of acetate into cholesterol and fatty acids (Table V). There was no interference with acetate incorporation into

TABLE V

Biosynthesis of cholesterol, fatty acids, and acetoacetic acid by liver homogenates from guinea pigs starved for 25 hours

Animal	Addition to incubation mixture	mμmoles acetate-1-C^{14} incorporated			μg aceto-acetic acid produced
		Cholesterol	Fatty acids	Acetoacetic acid	
7	Control	<0.1	0.3	41.0	225.6
	G-6-P (30 μmoles)	<0.1	0.3	36.5	225.2
	G-6-P (30 μmoles) + TPN (4 μmoles)	<0.1	0.3	41.1	209.1
8	Control	<0.1	0.2	46.4	108.6
	G-6-P (30 μmoles)	<0.1	0.3	46.5	122.5
	G-6-P (30 μmoles) + TPN (4 μmoles)	<0.1	0.3	48.3	113.3
9	Control	<0.1	0.4	19.7	215.7
	DL-Isocitrate (21 μmoles)	<0.1	0.5	29.4	205.7
	DL-Isocitrate (21 μmoles) + TPN (4μmoles)	<0.1	0.6	40.2	276.1

NOTE: Incubation procedure as in Table I; 5 μmoles DPN, 2 μmoles ATP, 1.32 μmoles acetate-1-C^{14} added to all samples.

acetoacetate, and total acetoacetate production showed some increase. It has been reported that addition of G-6-P and TPN to liver homogenates from alloxan diabetic rats fully restores cholesterogenesis and lipogenesis (12). On that basis, both G-6-P and isocitrate in combination with 4 μmoles TPN were added to starved liver homogenates but, as seen in Table V, failed to restore biosynthesis in the starved system. On the other hand when butyrate-1-C^{14} was incubated with starved liver homogenate, lipogenesis was partly restored by isocitrate and TPN (Table VI). The explanation of this may be in the observations by Long and Porter (26) that butyryl CoA may be incorporated directly into long-chain fatty acids and that only some of it is first cleaved to acetyl CoA.

TABLE VI

Biosynthesis of cholesterol, fatty acids, and acetoacetic acid by liver homogenates from guinea pigs

Condition of experiment	Addition to incubation mixture	mμmoles butyrate-1-C^{14} incorporated			μg aceto-acetic acid produced
		Cholesterol	Fatty acids	Acetoacetic acid	
Normal animal	Control	1.2	0.7	92.7	89.3
	DL-Isocitrate (30 μmoles) + TPN (4 μmoles)	0.2	33.9	111.3	48.5
Animal starved 25 hours	Control	<0.1	0.1	97.5	99.2
	DL-Isocitrate (30 μmoles)	<0.1	0.7	—	93.5
	DL-Isocitrate (30 μmoles) + TPN (4 μmoles)	<0.1	1.9	100.2	62.5

NOTE: Incubation procedure as in Table I; 5 μmoles DPN, 2 μmoles ATP, 0.85 μmoles butyrate-1-C^{14} added to all samples.

Cholesterol and fatty acid biosynthesis are readily restored when animals are fed following a 36-hour starvation period (Table VII). However, orally administered dextrose did not restore the in vitro biosynthesis of cholesterol

TABLE VII

Biosynthesis of cholesterol, fatty acids, and acetoacetic acid by liver homogenates from guinea pigs starved 36 hours and put back on feed for time indicated

Length of time on feed after starvation period	mμmoles acetate-1-C^{14} incorporated			μg aceto-acetic acid produced
	Cholesterol	Fatty acids	Acetoacetic acid	
12 hours	0.4	2.1	54.0	88.3
24 hours	5.7	3.3	43.4	62.6
36 hours	2.3	6.2	46.0	90.8

NOTE: Incubation procedure as in Table I; 5 μmoles DPN, 2 μmoles ATP, 1.32 μmoles acetate-1-C^{14} added to all samples.

and fatty acids (Table VIII). This is in agreement with observations by Scaife and Migicovsky (22), who used rat liver homogenates, but is contrary to observations by Tomkins and Chaikoff (27) and Lyon *et al.* (28), who noted that a single oral dose of 5 g dextrose to fasted rats completely restored lipogenesis in liver slices and that cholesterol synthesis was completely restored with four oral doses of 4 g dextrose at 6-hour intervals.

TABLE VIII

Biosynthesis of cholesterol, fatty acids, and acetoacetic acid by liver homogenates from guinea pigs starved for 40 hours. Oral dosing as indicated

Condition of experiment	mμmoles acetate-1-C^{14} incorporated			μg aceto-acetic acid produced
	Cholesterol	Fatty acids	Acetoacetate	
Starved 40 hr	<0.1	0.1	28.0	126.2
Starved 40 hr	<0.1	0.4	78.2	117.6
Starved 40 hr, 5 g dextrose administered in 12-hr poststarvation period	<0.1	0.1	30.1	81.2
Starved 40 hr, 7 g dextrose administered in 23-hr poststarvation period	<0.1	0.7	68.8	76.5
Starved 40 hr, 7 g dextrose administered in 25-hr poststarvation period	<0.1	0.1	35.0	65.7

NOTE: Incubation procedure as in Table I; 5 μmoles DPN, 2 μmoles ATP, 1.32 μmoles acetate-1-C^{14} added to all samples.

Acetate incorporation into acetoacetate was not blocked in homogenates from starved animals. This is in agreement with the suggestion made by Migicovsky (29), Scaife and Migicovsky (6), and Bucher *et al.* (30) that synthesis in starved animals is blocked at the level of β-hydroxy-β-methyl glutaryl CoA and that production of acetoacetate is not suppressed.

Liver homogenates from starved guinea pigs in no instance contained over 10 μg acetoacetate before incubation. It follows that the intact guinea pig

utilizes acetoacetate as rapidly as it is produced and that acetoacetate does not accumulate in vivo even after 40 hours' starvation.

In vitro production of acetoacetate by liver homogenates from starved guinea pigs was increased in some experiments but this was not a consistent finding. The fact that liver homogenates from starved guinea pigs do not accumulate large quantities of acetoacetate is not surprising since oxidation and CO_2 production are not impaired in liver systems of fasting animals (22, 31, 32). Considerably more acetate is oxidized than is incorporated into lipids or sterols and impaired biosynthesis alone would not necessarily lead to a large accumulation of acetoacetate.

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A MICROMETHOD FOR THE DETERMINATION OF SULPHATE BY FLAME PHOTOMETRY

G. A. ROBINSON

Abstract

A micromethod for the determination of 0.1 to 3.5 μg of sulphate is described. Acidified barium chloride solution is added to samples containing free and esterified sulphate, and the resultant mixtures are dried at 95° C. Soluble materials are redissolved with a 90% solution of acidified ethanol, 0.1 *N* for hydrochloric acid, containing 250 μg lithium per milliliter. After addition of the ethanol solution, approximately eight hours are required for the establishment of equilibrium (as indicated by a radiosulphur label) between solid and dissolved barium sulphate. Barium present in solution is then determined quantitatively by flame photometry, with the ethanol–lithium medium increasing the primary emission of the barium by 15 times. The sulphate content of the sample is read from a standard curve. Estimations on microgram quantities of some organic and inorganic sulphur-containing compounds are given.

Introduction

The quantitative determination of sulphate resulting from the oxidation (1, 2) or hydrolysis (3) of organic materials has been effected by several basic methods. Gravimetry of barium sulphate (4, 5) or benzidine sulphate (1) precipitates, titration of the acid produced (6, 7), nephelometry (8, 9), and manometry (10) have been reported. Colorimetry of a derivative of the benzidine sulphate precipitate has also been used (3, 11, 12). An indirect method was referred to by Nachtrieb (13), in which sulphate was precipitated by a strontium solution of known concentration. The flame emission of the strontium remaining in solution was then measured. Strickland and Maloney (14) reported a similar procedure in which barium (20 meq/liter) was used as the precipitating agent.

In the present method, barium (0.15 meq/liter) was employed, even though strontium has been detected by flame photometry in concentrations of the order of 0.1 times those for barium (13, 15, 16). But the solubility of strontium sulphate, 113 μg per ml water (17), was unsuitable for a micromethod. Using barium chloride, determinations have been made in this laboratory on materials containing 0.1 to 3.5 μg sulphate per sample. This range was comparable to the 10 μg lower limit reported by Nechaeva (12) and Dodgson and Spencer (3).

Reagents

All solutions were prepared with doubly distilled water and "sulphate-free" reagents. Glassware was cleaned in $KMnO_4$ and HCl solutions, rinsed several times with tap water and twice with distilled water, then given a final rinse with doubly distilled water.

Barium solution A (1 mg barium/ml of solution): 1.778 g $BaCl_2.2H_2O$ in 1 liter.

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Barium solution B (20 μg barium/ml of 0.5 *N* HCl): 20 ml of barium solution A + 41.4 ml 37% HCl reagent, made up to 1 liter.

Ethanol solution A: 5 ml of barium solution A + 1.53 g LiCl + 8.27 ml 37% HCl + 900 ml redistilled ethanol, made up to 1 liter with doubly distilled water.

Ethanol solution B: 1.53 g LiCl + 8.27 ml 37% HCl + 900 ml redistilled ethanol, made up to 1 liter.

Hydrochloric acid, 0.5 *N*: 41.4 ml 37% reagent/liter of solution.

Sulphate solution A (1 mg sulphate/ml): 1.37 g $(NH_4)_2SO_4$ in 1 liter.

Sulphate solution B (2 μg sulphate/ml): 2 ml of sulphate solution A, made up to 1 liter.

Procedure

Equal volumes of test solution (e.g. 1 ml) were delivered into each of six 10 × 75 mm Pyrex culture tubes. Then 0.25 ml 0.5 *N* HCl was added to each of tubes Nos. 1, 2, 5, and 6 and 0.25 ml of barium solution B to each of the remaining two tubes. All tubes were dried at 95° C. When cool, each of tubes Nos. 3, 4, 5, and 6 received 1 ml of ethanol solution B, was shaken, stoppered, and stored at room temperature overnight. Immediately before the flame photometry, 0.25 ml of ethanol solution A was added to each of tubes Nos. 1 and 2.

A Hilger Uvispek (Model H700, quartz prism), flame attachment (H868), and a Brown-Honeywell Electronik recorder were used for the flame photometry. Adjustments for maximum sensitivity were made with oxygen at 30 p.s.i., hydrogen 20 p.s.i., wavelength 8500 Å, and a slit width of 0.50 mm. If visible particles (carbon flakes, organic matter) were present in the samples, the contents of the tubes were filtered through small plugs of acid-washed cotton. Otherwise the feed tube of the atomizer may have become occluded. Solutions were sprayed for 15 to 20 seconds each, with minimal intervals between readings. The performance of the instrument was checked periodically using ethanol solutions A and B as chart calibration solutions. The mean of the tracings recorded for the contents of tubes Nos. 1 and 2 represented an emission of 100%. The 0% limit for the sample was established using tubes Nos. 5, 6. Per cent emissions for tubes Nos. 3, 4 were then obtained, and the quantity of sulphate present in the sample was estimated from a standard curve.

The standard curve (Fig. 1) was derived in a similar manner. The 100% and 0% limits were established in quadruplicate on 1-ml volumes of doubly distilled water. Intermediate values were obtained from duplicate volumes (up to 2 ml) of sulphate solution B. These sulphate standards were dried and redissolved in 1 ml of doubly distilled water before addition of barium solution B.

Notes on the Procedure

Initial Preparation

For test materials, solutions (Table I) and wet precipitates in which the sulphate-containing compounds were freely soluble in dilute hydrochloric acid

TABLE I

Apparent sulphate content of some test solutions

Material	Quantity per sample	% emission	Apparent sulphate (μg/sample)
Chondroitin sulphate	20 μg	18	2.3
Doubly distilled water	1 ml	100	0
Equilin sulphate	6 μg	71	0.8
Ethanol (redistilled)	0.5 ml	100	0
Heparin	6 μg	40	1.7
Methyl blue	6 μg	99	0
NaCl	100 μg	103	0
$Na_3PO_4.12H_2O$	40 μg	103	0
Na_2SO_3	6 μg	94	0.2
$(NH_4)_2SO_4$	3 μg	22	2.2
Platelets (calf)	9.4×10^8	5.2	2.8
Serum (calf)	0.015 ml	68	0.9
Sulphamethazine	6 μg	102	0
Tap water	0.2 ml	11	2.6

solution were used. When tubes containing barium and sulphate standards were stoppered and stored at room temperature for 14 days, the barium concentration was reduced in proportion to the weight of standard sulphate present even though no precipitate could be seen. The time required for a single series of determinations was reduced to 1 day by drying the samples after addition of the barium reagent. Ninety per cent ethanol was used for redissolving the samples since the solubility of $BaSO_4$ in a 0.1 *N* solution of HCl in water was found to be 10.2 μg per ml. Absolute ethanol was unsuitable; $BaSO_4$ is very sparingly soluble in this medium, but so are other barium salts (e.g. $BaCl_2$ approximately 40 μg/ml). The acidic condition prevented precipitation of barium as carbonate, hydroxide, or phosphate. Co-precipitation by other ions (4, 5) or solubility of $BaSO_4$ as a function of particle size (18) were not considered to have been a problem.

The location of sulphate during the initial preparation was indicated by radiosulphur. Standards were prepared from a sulphate solution containing 0.05 μc $S^{35}O_4$ per μg of sulphate. Barium was added, and the tubes dried. Two strips of Kodak contrast process ortho film (10 mm × 100 mm, with one rounded end) were introduced into each tube. Twenty-four hours later, the strips were removed and developed. Films had darkened at the edge of the rounded ends and for a few millimeters along the sides. Consequently, most of the sulphate was assumed to have been spread over the bottom curvature of the tubes. Ethanol solution B (0.25 ml) was then added to each tube. Radioactivity of this fluid was measured (NRD DR-14 gas-flow counter) at various time intervals over a 24-hour period. The concentration of S^{35} and hence of sulphate in the ethanol solution increased for 6 to 8 hours, after which a constant level was approximated. Immediately after removing fluid from a tube for counting, the tube was rinsed with 1 ml of doubly distilled water, dried at 95° C, cooled, and the radioautography repeated. Darkening of the film strips was less than previously, but the distribution of radioactive sulphate was unchanged.

Flame Photometry

Willard, Merritt, and Dean (15) stated that the infrared band for barium at 8500 Å was more intense than either of the major green bands at 5500 Å and 5200 Å. Watanabe and Kendall (16) concluded that the most sensitive band for the barium–chlorine system was at 873 mμ, although 553.6 mμ was reported as showing the same emission intensity for a smaller slit width. In the present investigation, approximately twice the sensitivity was observed for the infrared (8200–8700 Å) as for the green region (5200–5600 Å). In addition, the green wavelengths were influenced both by intermittent sodium emissions and by copper oxide flakes from the baffle plates. Calcium (5560 Å) also presented an interference problem. For the infrared region some interference from K_2, CN, and NaK molecules was possible (19). Caesium (8520 Å) would have been a serious contaminant, but this element was not likely to be found in most biological materials.

A slit width of 0.50 mm was required to balance the Uvispek at 8500 Å when spraying ethanol solution B. The total band width of approximately 265 Å included bands for barium–chlorine (8420.8 Å) and barium–fluorine (8571.5 Å), but avoided iron–oxygen at 8297 Å and water at 8916 Å (19).

Ethanol and lithium were used in the dissolving fluid because emission intensities for water solutions containing 5 μg Ba/ml were not sufficient to produce satisfactory recordings (Table II). Alkali chlorides increased the

TABLE II

Intensity of barium–chlorine flame emission at 8500 Å

Solvent	Background emission (mm)*	Barium emission, 2 μg Ba/ml solvent (mm)*	Relative barium emission intensity†
Doubly distilled water	0	4.8	1
0.1 *N* HCl	−1.4	11.2	2.3
90% Ethanol	−25.2	17.1	3.6
250 μg Li/ml water	58.0	26.9	5.6
250 μg Li/ml 90% ethanol	133.0	57.7	12.1
250 μg Li/ml 90% ethanol, 0.1 *N* for HCl	137.0	71.7	15.0

*Number of millimeters recorder pen traverses on chart paper.
†Emission for 2 μg Ba/ml doubly distilled water equals 1.

emission, probably by formation of barium complexes with emission energies greater than for the barium–chlorine system. (This observation was analogous to the positive interaction described by Bianchi (20) for Na, K; Ca, Mg.) Lithium did not produce as great an increase in the barium emission as did equivalent amounts of sodium and potassium, but the low molecular weight of LiCl and the weak background emission of this salt at 8500 Å permitted a relatively high concentration without excessive encrustation of the burner holes. Chart recordings were unsatisfactory for the caesium–barium system because of the high primary emission of caesium at this wavelength. Ethanol resulted in more efficient operation of the atomizer and spray chamber; consequently a greater rate of delivery of barium and lithium to the flame produced an apparent increase in emission intensities for this system.

Interference with the flame photometry by some foreign ions was expected. In particular, sodium from the walls of the tube and sodium and potassium from biological samples produced an apparent increase in the barium content of the dissolving fluid. Using six tubes for each sample compensated for this interference in that 0% and 100% emissions were established individually. The 0% tubes accounted for a change in background light intensity and the 100% tubes for direct interference with the barium emission.

Results

The standard curve (Fig. 1) is primarily the sum of a precipitation component which relates the weight of barium precipitable as $BaSO_4$ to the weight of sulphate added, and a solubility component which represents the solubility of

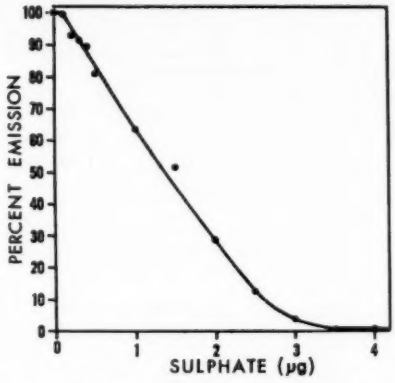

FIG. 1. The curve relating the flame emission of barium remaining in solution after addition of 5 μg barium (as chloride) to the micrograms of sulphate present in the sample. The solvent medium was 90% ethanol, 0.1 *N* for HCl. The barium emission at 8500 Å was intensified by the addition of LiCl (382 μg/ml) to the solvent.

the salt in the solution used. The useful range for the method as described is approximately 0.1 to 3.5 μg of sulphate per sample. The curve is non-functional below 0.1 μg, as all of the $BaSO_4$ formed remains in solution.

Sulphate estimations for chemicals in Table I are for laboratory shelf preparations. Should a particular application of the method require the absence of ethanol or lithium, an alternative procedure has given good results. The weights (or volumes) of standards and samples are increased by a factor of four; 1-ml volumes of barium solution B and of 0.5 *N* HCl are used in place of 0.25 ml, and ethanol solutions A and B are replaced by barium solution C (20 μg Ba/ml of 0.1 *N* HCl) and 0.1 *N* HCl respectively. The standard curve so obtained has a useful range of 1.5 to 15 μg sulphate per sample.

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EFFECTS OF DIET ON INCORPORATION OF ACETATE-1-C^{14} INTO CHOLESTEROL BY RAT LIVER SLICES[1]

K. K. CARROLL

Abstract

The rate of incorporation of acetate-1-C^{14} into cholesterol by rat liver slices decreased when animals were transferred from a commercial chow diet to semisynthetic diets. A greater decrease was observed when the test diet contained oleic acid or olive oil than when it contained higher homologues of oleic acid, or preparations of cerebrosides. The decrease was not affected by the presence or absence of fat-soluble vitamins in the test diet containing oleic acid. Amphenone B affected acetate incorporation into liver cholesterol differently depending on whether it was fed in a chow diet or a semisynthetic diet. The incorporation of acetate into liver fatty acids of rats fed various diets usually increased or decreased with incorporation into cholesterol but differences between groups were less marked. Incorporation of acetate into cholesterol by intestinal mucosa was relatively insensitive to changes in diet.

Introduction

Previous work has shown an increased rate of incorporation of acetate-1-C^{14} into cholesterol by liver slices or homogenates of rats fed rapeseed oil or its major fatty acid, erucic acid (1, 2), and a similar effect was observed in in vivo experiments with rats (1). The present studies are concerned with effects on acetate incorporation of feeding related fatty acids, cerebroside preparations, or amphenone B, all of which have been shown to alter cholesterol metabolism in intact rats (3–6).

In these experiments young rats raised on a commercial chow diet were transferred to purified diets containing the substances under test and after 1 to 4 weeks the animals were killed and the rate of incorporation of acetate into cholesterol by liver slices in vitro was measured. During the course of the work it was found that the transfer of rats from the chow diet to semisynthetic diets was followed by a decrease in the rate of acetate incorporation into cholesterol. The results obtained are discussed in the light of this unexpected finding.

Experimental

These experiments were performed on young male Sprague–Dawley rats caged in groups of three and fed experimental diets for 1 to 4 weeks before they were killed. The diets had the following composition, expressed as parts by weight: casein (purified) 22, dextrose 52, fatty acid or cerebroside preparation 15, salt mixture 5, and cellu flour 5. The diets contained an adequate supplement of water-soluble vitamins but fat-soluble vitamins were added only when indicated. The sources of dietary materials have been described previously (7). The cerebroside preparation (WT-5) was kindly supplied by Dr. David Klein of the Wilson Laboratories, Chicago. The methyl esters of cerebroside fatty

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Contribution from Collip Medical Research Laboratory, University of Western Ontario, London, Ontario.

acids were prepared by treatment of this preparation with sulphuric acid in methanol as described by Carter *et al.* (8). Other fatty acids were obtained as follows: oleic acid — Nutritional Biochemicals, Cleveland, Ohio; eicosenoic acid — Dr. B. M. Craig, Prairie Regional Laboratory, Saskatoon, Sask.; erucic acid — Archer-Daniels-Midland Co., Cleveland, Ohio. Nervonic acid was prepared synthetically (9). Oleic acid was purified by distillation *in vacuo* and erucic acid by two recrystallizations from methanol at 0° C (2). Amphenone B was obtained through the courtesy of Dr. C. Walter Murphy, CIBA Ltd., Montreal. Two types of chow diet were used: Master Fox Breeder Starter Ration from Toronto Elevators, Limited, Toronto, Ontario, and Purina Fox Chow Meal from the Ralston Purina Company, Woodstock, Ontario. These will be referred to as Master Meal and Purina respectively. The analyses of these diets were given as: protein 20% min., fat 3% min., and crude fiber 5% max.

Liver slices or scrapings of intestinal mucosa (750 mg) were incubated for 3 hours at 37° C in an atmosphere of oxygen with 5 ml of Krebs–Ringer phosphate buffer (pH 7.1) containing acetate-1-C^{14}. Cholesterol and fatty acid fractions were extracted and counted as in earlier experiments (2).

Results

Results of the studies with liver slices are presented in Table I. In the first experiment groups of rats were fed diets containing the homologous monounsaturated fatty acids, oleic (C_{18}), eicosenoic (C_{20}), erucic (C_{22}), and nervonic (C_{24}). Because of the limited amounts of eicosenoic and nervonic acid available the groups were restricted to three rats each and diets were fed for only 1 week. The results show that liver slices from rats fed eicosenoic, erucic, or nervonic acids incorporated similar amounts of labelled acetate into cholesterol, while slices from rats fed oleic acid incorporated less.

In a second experiment the effects of feeding amphenone B, a cerebroside preparation, or the fatty acid esters derived from the cerebroside preparation were investigated. Amphenone B was added to a semisynthetic diet containing 15% oleic acid and the same diet without amphenone was fed as a control. In this experiment the amount of acetate incorporated into cholesterol was 10 to 20 times greater in the experimental groups than in the control group. However, incorporation in the control group was much less than that observed in experiment 1 where the same diet was fed for only 7 days. It was also our impression from earlier experiments (2) that when rats were fed purified diets containing no fat or the common C_{16} or C_{18} fatty acids, the observed acetate incorporation into cholesterol was low in comparison with results from other laboratories. Since other workers have in most cases used rats fed commercial chow diets, it was decided to compare the effect, on acetate incorporation, of feeding chow diets versus feeding semisynthetic diets.

In experiment 3, groups of animals were fed semisynthetic diets containing either 15% oleic acid or 15% olive oil for 25 days and the acetate incorporation was compared with that for animals of the same approximate age and weight but fed either Master Meal or Purina. Acetate incorporation into cholesterol

TABLE I

Incorporation of acetate-1-C^{14} into cholesterol and fatty acids by liver slices

Expt. No.	Type of diet	No. of rats	Body wt.	Days on diet	Acetate* concn., *M*	Cholesterol		Fatty acids	
						Weight, mg/g tissue	Activity†	Weight, mg/g tissue	Activity†
1	15% oleic acid	3	162	7	.0002	2.4±0.1‡	2.8 ±0.2‡	34±0.4‡	4.8±0.5‡
	15% eicosenoic acid	3	165			2.6±0.3	6.6 ±1.4	36±0.7	5.2±0.4
	15% erucic acid	3	142			2.9±0.3	6.6 ±1.0	32±0.6	13.0±2.3
	15% nervonic acid	3	152			2.5±0.2	8.0 ±1.1	34±2.8	8.2±1.4
2	15% oleic acid	6	212	25	.0002	3.9±0.4	0.25±0.05	37±1.4	2.2±0.3
	15% oleic acid 0.25% amphenone B	6	135			10.7±0.4	2.0 ±0.3	77±6.0	3.2±1.0
	15% cerebrosides	6	223			1.8±0.2	3.7 ±0.6	31±0.8	2.6±0.8
	15% cerebroside fatty esters	6	197			1.8±0.1	4.6 ±0.9	32±0.8	4.2±1.5
3	15% oleic acid	3	205	25	.0002	3.5±0.4	0.76±0.3	37±3.6	2.3±0.4
	15% olive oil	3	208			4.6±0.6	0.59±0.4	34±2.1	3.8±0.7
	Master Meal	12	195			2.1±0.1	10.4 ±1.1	29±0.5	8.0±1.6
	Purina	5	213			2.2±0.1	5.7 ±1.2	31±0.4	5.3±0.9
4	15% oleic acid§	3‖	200	22	.0002	2.7±0.1	1.0 ±0.3	38±5.2	3.2±0.7
		3	195		.001	2.7±0.1	0.64±0.1	36±1.2	2.8±0.4
		3	196		.004	2.5±0.3	0.54±0.1	41±1.5	2.8±0.5
		3	197		.008	2.6±0.1	0.37±0.1	37±2.6	2.0±0.2
	Master Meal	3‖	189		.0002	1.7±0.3	10.0 ±3.6	32±2.0	16.0±0.9
		3	192		.001	1.9+0.2	4.9 ±1.0	32±0.9	11.3±3.1
		3	190		.004	2.4±0.2	3.4 ±0.2	30±1.1	9.2±2.3
		3	189		.008	1.8±0.2	5.1 ±2.2	30±0.7	8.4±2.4
5	15% oleic acid	3	131	7	.0002	2.2±0.2	1.7 ±0.5		
	15% oleic acid§	3	137			2.1±0.1	1.8 ±0.1		
	Master Meal	3	140			1.7±0.1	11.0 ±2.8		
6	15% oleic acid	3	173	14	.0002	2.7±0.1	1.3 ±0.4		
	15% oleic acid§	3	170			2.5±0.1	0.8 ±0.2		
	Master Meal	3	154			1.4±0.1	7.1 ±1.7		

TABLE I (*Concluded*)

Incorporation of acetate-1-C^{14} into cholesterol and fatty acids by liver slices

Expt. No.	Type of diet	No. of rats	Body wt.	Days on diet	Acetate* concn., *M*	Cholesterol		Fatty acids	
						Weight, mg/g tissue	Activity†	Weight, mg/g tissue	Activity†
7	Master Meal	6	205	22	.0002	2.3±0.2	7.5 ±1.1	27±1.6	5.9±1.3
	Master Meal 0.25% amphenone B	6	154			3.3±0.2	1.6 ±0.5	46±1.7	2.3±0.5

*The acetate in each incubation flask had a total activity of approximately 1×10^6 counts/minute.
†Activity is expressed as per cent of counts in added acetate-1-C^{14} recovered in cholesterol or fatty acids.
‡Standard error of the mean.
§Only four rats per diet were used in this experiment and liver slices from each rat were incubated with three different concentrations of acetate.
[¶]These diets contained 20 mg of vitamin A acetate, 5.5 mg of vitamin D, 110 mg of DL-α-tocopherol acetate, 37.5 mg of vitamin K, and 15 ml of corn oil per kg.

was much lower in animals fed the purified diets. Experiment 4 shows that this difference was maintained over a wide range of acetate concentration in the incubation medium. Further comparisons between animals fed semisynthetic diets containing oleic acid and those fed Master Meal were made in experiments 5 and 6. A decrease in the amount of acetate incorporated into cholesterol was very apparent both after 7 and 14 days of feeding the semisynthetic diet. The presence or absence of fat-soluble vitamins in this diet made no difference to the result obtained.

Experiment 7 was carried out to examine the effect on acetate incorporation into cholesterol of feeding amphenone in a chow diet, since in earlier experiments amphenone increased the concentration of cholesterol in plasma and liver of rats when it was fed in a purified diet but not when fed in a chow diet (6). The results showed that acetate incorporation was depressed by addition of amphenone to the chow diet in contrast to the result of experiment 2 where amphenone was fed in a semisynthetic diet.

The concentration of cholesterol in the liver was generally somewhat higher in animals on semisynthetic diets than in those on chow diets. This difference was most noticeable in groups fed for 25 days on diets containing oleic acid or olive oil and was not observed in groups fed cerebroside preparations (experiments 2 and 3). As in previous experiments, a marked elevation in liver cholesterol was observed when amphenone was fed in a purified diet but only a small elevation when it was fed in a chow diet (experiments 2 and 7).

The concentration of liver fatty acids was also slightly higher in animals on semisynthetic diets. It approximately doubled when amphenone was fed either in a purified or a chow diet and livers of animals fed amphenone appeared fatty on gross inspection. Incorporation of acetate into fatty acids tended to parallel incorporation into cholesterol but differences between groups fed different diets were in general less marked than those observed in the cholesterol results. The high value observed for the group fed erucic acid in experiment 1 differs from previous results (2) and is probably not typical.

A few further experiments were carried out on incorporation of labelled acetate into cholesterol and fatty acids by scrapings of intestinal mucosa and the results are presented in Table II. As observed previously (2), incorporation

TABLE II

Incorporation of acetate-1-C^{14} into cholesterol and fatty acids by intestinal mucosa

Expt. No.	Type of diet*	No. of rats	Body wt.	Cholesterol		Fatty acids	
				Weight, mg/g tissue	Activity†	Weight, mg/g tissue	Activity†
1	15% oleic acid	6	196	2.7±0.2‡	2.4±0.4‡	34±3.0‡	2.0±0.2‡
	15% oleic acid 0.25% amphenone	6	139	2.7±0.1	1.2±0.1	34±2.2	1.6±0.1
	15% cerebrosides	6	213	2.5±0.1	2.0±0.3	30±1.0	2.6±0.2
	15% cerebroside fatty esters	6	184	2.7±0.2	2.2±0.3	31±0.9	2.7±0.2
2	15% oleic acid	3	205	3.2±0.1	3.4±0.2	35±2.2	2.4±0.6
	15% olive oil	3	208	2.7±0.2	2.0±0.1	40±4.3	2.2±0.3
	Master Meal	6	203	2.7±0.1	1.4±0.1	25±0.9	2.6±0.3

*The diets were fed for 25 days.
†Activity is expressed as per cent of counts in added acetate-1-C^{14} recovered in cholesterol or fatty acids. Each incubation flask contained 0.0002 *M* sodium acetate having a total activity of approximately 1×10^6 counts/minute.
‡Standard error of the mean.

by intestinal mucosa was relatively unaffected by changes in diet. However, the amount of acetate incorporated into cholesterol was significantly lower in the group fed amphenone (experiment 1) and in the group fed Master Meal (experiment 2).

Discussion

These experiments have revealed differences in incorporation of acetate into cholesterol by liver slices of rats fed different experimental diets. However, incorporation by slices from rats fed test diets was almost always lower than that observed for rats fed chow diets. Since the animals were all maintained on a chow diet (Master Meal) prior to being fed experimental diets, it appears that the transfer of animals to the type of semisynthetic diet used in this work was generally followed by a decreased incorporation of acetate into cholesterol. Since in earlier experiments (2), the incorporation was as low in animals on a fat-free diet as in those on diets containing fatty acids, this effect does not seem to be related specifically to the fat component of the semisynthetic diets.

This difference between rats fed semisynthetic and chow diets complicates interpretation of results of experiments in which specific components were added to the test diets to study their effects on acetate incorporation. It was previously concluded that feeding of erucic acid to rats stimulated acetate incorporation into cholesterol by liver slices in vitro (2) and this conclusion seems justified since Wood and Migicovsky obtained a similar result by feeding erucic acid mixed with a chow diet (1). However, these workers also found that acetate incorporation was increased in rats fed a chow diet containing oleic acid.

The results obtained with amphenone B illustrate the difficulties of determining the effect of a single dietary component on cholesterol metabolism. Amphenone fed to rats in a purified diet stimulated acetate incorporation into cholesterol while amphenone fed in a chow diet inhibited acetate incorporation (Table I, experiments 2 and 7). These results, although puzzling, are in accord with results of earlier studies in which rats fed amphenone in purified diets showed increased concentrations of cholesterol in liver and plasma while rats fed amphenone in Master Meal had nearly normal levels of liver and plasma cholesterol (6).

It has been suggested that the rate of cholesterol synthesis by the liver is controlled by a homeostatic mechanism in which a rise in liver cholesterol depresses hepatic synthesis so that a rise in total body cholesterol is minimized (10). Gould (11) emphasized that the regulatory factor is probably free cholesterol rather than ester cholesterol but recent papers continued to interpret liver cholesterol biosynthesis in relation to total cholesterol concentration of the liver (12, 13). However, in our experiments with amphenone fed in a semisynthetic diet, an increased concentration of liver total cholesterol was associated with a much higher rate of incorporation of acetate into cholesterol than that observed in controls. Although only total liver cholesterol was measured in these experiments, it was shown earlier that increases in liver cholesterol which resulted from feeding amphenone in purified diets were confined entirely to the ester cholesterol fraction (6).

Bucher *et al.* (14) have suggested that comparisons of cholesterol biosynthesis by different liver homogenates will be more reliable if the concentration of labelled substrate in the incubation medium is not a limiting factor. On the other hand, Emerson and Van Bruggen (15) feel that if labelled substrate is present in amounts exceeding tracer concentration, it may influence the reaction being studied and the incorporation of label may not quantitatively reflect rates of reaction prevailing prior to its introduction. In view of these considerations, liver slices from rats fed a chow or a purified diet were incubated in media containing varying concentrations of acetate but the same total amount of radioactivity. The results (Table I, experiment 4) showed that the difference in incorporation rate between animals fed the different diets was maintained over a wide range of acetate concentrations.

Differences in cholesterol metabolism between rats fed chow diets and rats fed purified diets have also been reported from other laboratories. Portman and his associates (16–18) have found that rats fed chow diets excrete three- to four-fold greater amounts of bile acids than rats on semisynthetic diets. Substitution of corn starch for sucrose in their purified diet caused a significant increase in bile acid excretion. Mosbach *et al.* (19) reported profound differences in acetate incorporation into cholesterol between rats fed chow diets and rats fed semisynthetic diets. Christophe and Mayer (20), however, found little difference in the in vivo incorporation of labelled acetate into cholesterol. It may also be pertinent that alterations in carbohydrate metabolism have been observed in liver slices of rats transferred from a chow diet to semisynthetic diets (21).

In their recent review on the dietary regulation of serum cholesterol, Portman and Stare (22) conclude that the interaction of dietary factors must be considered in assessing their effects on cholesterol metabolism. They also suggest that proper evaluation of the effect of any single factor can only be attained by studying it under a wide range of conditions. The results of the present study add emphasis to these conclusions.

Acknowledgments

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ELECTROPHORETIC PROPERTIES OF CRYSTALLINE GLOBULIN FROM CUCURBIT SEEDS[1]

DAVID GENE ANDERSON,[2] A. G. MCCALLA, AND D. R. MCCALLA[3]

Abstract

Crystalline globulins from squash and pumpkin seed yielded two distinct electrophoretic components at pH 4.8 but those from cucumber and watermelon seed yielded only one. Each yielded a single component at pH 4.6 but at pH values below 3.5 there was a tendency for a second component to appear. At pH 2.3 all showed two components although separation was not complete nor was it uniform among species. There was a tendency for two components to appear at pH values above the isoelectric range.

The results support the conclusion that the globulins from closely related species differ less than those from more distantly related species. They suggest that the crystalline globulins are not each a single homogeneous protein.

Introduction

The sedimentation characteristics of crystalline globulins from cucurbit seeds were recently discussed by Anderson and McCalla (1). Electrophoretic analyses were made on many of the same preparations and are herewith reported.

Fuerst *et al.* (2) studied the crystalline globulin from squash seeds and found that, while a single component was present in most preparations, two distinct components were present at pH 4.7. The sharp change that occurred between pH 4.7 and lower values could not be explained on the basis of the information obtained.

Kretovich *et al.* (3) reported the separation of two electrophoretic components when the globulin from pumpkin seeds was prepared using Osborne's procedure (4), but four components when a diffusion desalting procedure of Zelenskii (5) was used. Unfortunately the original paper was not available to the writers. The suggestion by Kretovich *et al.* that the Osborne method caused denaturation does not appear to be supported by the results obtained by Fuerst *et al.* nor by the results of the present work.

So far as the writers are aware no other studies of this type have been carried out on cucurbit globulins.

Material and Methods

Details concerning the materials and methods of preparation are given in the earlier paper (1). Crystalline globulin was prepared from the seeds of squash (*Cucurbita maxima*), pumpkin (*Cucurbita pepo*), cucumber (*Cucumis sativus*), and watermelon (*Citrullus vulgaris*).

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Contribution from the Department of Plant Science, University of Alberta, Edmonton, Alberta, with financial assistance from the National Research Council of Canada. Based on part of a thesis submitted to the Faculty of Graduate Studies as a requirement for the M.Sc. degree.

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All preparations to be used for electrophoretic analyses were dialyzed for 3 days under static conditions or for at least 24 hours if the dispersion was kept in swirling motion. The large volume of buffer used in dialysis was later used in the electrophoretic apparatus.

All analyses were carried out in a Klett-Tiselius apparatus using the schlieren-scanning technique described by Longsworth and McInnes (6). Current was controlled by a voltage-regulated power supply with which variation could be controlled to $\pm 0.5\%$ between 0 and 200 milliamperes.

All analyses were made at 20° C since the globulins are almost insoluble at 4° C. Fuerst *et al.* (2) compared results obtained at the two temperatures and used 20° C throughout the study. Good results were obtained with low currents and longer runs, but difficulties with convection arose at higher current levels.

While the electrophoretic diagrams constitute the more important part of the results, mobilities were calculated for all runs. Mobility was calculated using the formula given by Alberty (7). Where skewing occurred, measurement was made at the maximum ordinate.

Results

The electrophoretic diagrams obtained with the globulin from the seed of the four species at pH 4.8 and at pH values from 4.1 to 4.5 are presented in Fig. 1. The results for the squash globulin agree with those obtained by Fuerst *et al.* The clear separation of two components at pH 4.7 and 4.8 (*a*) has been obtained repeatedly. Fuerst's diagram showing only one component was obtained at pH 3.9 but the results of the present study show that the single component occurs at all pH levels from 3.9 to 4.6. The diagrams in Fig. 1(*b*) are representative of all those obtained over this range.

It is obvious that the globulin from pumpkin seed behaves in a similar manner. The diagrams in Fig. 1(*d*) are representative of all single component diagrams obtained in the study. There appears to be a somewhat larger proportion of the protein in the slower component than with squash globulin but this is the only difference.

The behavior of the globulin from cucumber and watermelon, on the other hand, is quite different in that a single component was found at all pH values between 3.9 and 4.7 or 4.8. There was no indication of separation into two components, although one set of diagrams obtained with cucumber globulin in 0.01 *M* acetate at pH 4.3 suggested that a second component might be present. At all other pH levels, each diagram clearly showed a single component. The faster component found in each of the squash and pumpkin globulins was absent.

Analyses were carried out at pH 2.3 in glycine–HCl buffers at several ionic strengths and in potassium chloride–HCl buffers at 0.5 *M*. All diagrams for all species, Fig. 2, strongly indicate more than one component, but clear separation of two components was obtained only in the ascending boundaries with squash and pumpkin globulin preparations. On the other hand, the proportion of the faster-moving component was much smaller than at pH 4.8. Although separa-

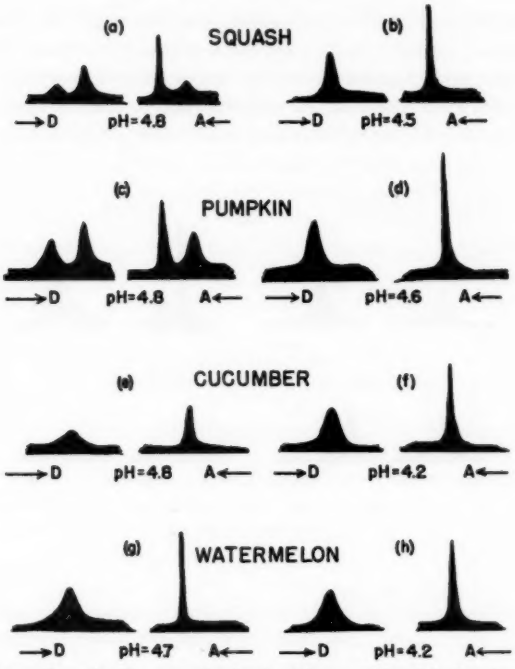

FIG. 1. Electrophoretic diagram obtained with cucurbit globulins in 0.01 *M* sodium acetate. Protein concentration from 0.37 to 0.62%, time from 3 to 4 hours, and current 1.3 to 2.3 volts/cm.

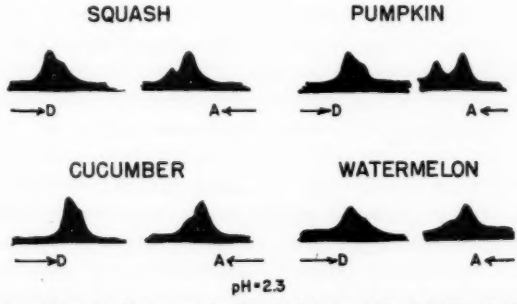

FIG. 2. Electrophoretic diagrams obtained with cucurbit globulins in 0.04 *M* glycine – hydrochloric acid, pH 2.3. Protein concentration approximately 0.4%, time from 3 to 4 hours, and current 1.1 to 2.3 volts/cm.

tion did not take place in the descending boundaries it is clear that there were two components present and that the faster one was present in smaller proportion than the slower one. Separation was not obtained in either boundary with cucumber or watermelon globulins at pH 2.3 but there was strong evidence that these proteins otherwise behaved in a manner similar to those from squash and pumpkin.

Fuerst (8) carried out analyses of squash globulin at pH levels well above the isoelectric range. At pH 9.9, two distinct components were present, but at pH 10.2 only one was obtained with no suggestion of a second. Much more extensive analyses were made in the present study. Fuerst's results for squash globulin were confirmed for descending boundaries, Fig. 3, and it was deter-

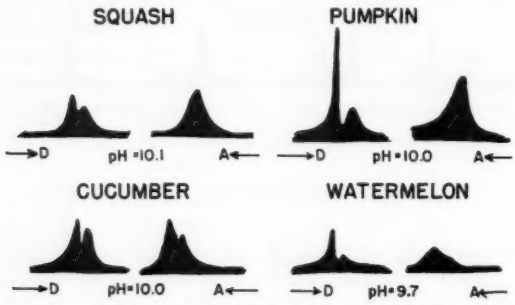

FIG. 3. Electrophoretic diagrams obtained with cucurbit globulins in 0.05 *M* glycine – sodium hydroxide buffers. Protein concentration from 0.27% to 0.57%, time 3 to 4 hours, and current 1.9 to 2.9 volts/cm.

mined that the sharp break in behavior appears to occur between pH 10.0 and 10.2, since all diagrams obtained above pH 10.2 showed only one component. Pumpkin globulin gave similar results, but cucumber and watermelon globulins were somewhat different in that symmetrical single component diagrams were not obtained at any pH level. The latter diagrams were not very satisfactory, however, and no reliable conclusions can be drawn from these experiments. The lack of symmetry in the diagrams makes interpretation very difficult.

Only a few of the many diagrams obtained in the study are presented in this paper, but all of the types of results are illustrated. Failure to get good agreement between ascending and descending boundaries is not uncommon and much has been written on this problem. It does not seem profitable to attempt to discuss the differences obtained since, so far as the authors are aware, no explanation yet offered explains satisfactorily the several differences in the results obtained. This is particularly true of the differences between the pairs of diagrams for squash and pumpkin globulin in Fig. 3 where descending boundaries clearly separated into two peaks but the corresponding ascending boundaries were merely skewed in the direction of migration. The diagrams were repeatedly obtained and cannot be considered to be the result of error. They may, however, be the result of inadequacy in the technique used.

Mobility rates were determined for a number of preparations from each of the species. The wide isoelectric range reduces the value of these studies since no measurements were possible between pH values of 4.8 and 8.2. From pH 3.9 to 4.8 the mobility decreased from 16×10^{-5} to 8×10^{-5} cm^2/volt/second, as one would expect with decreasing charge on the protein. At pH 2.3, however, the mobility was about the same as at pH 3.9 and below this it decreased.

The results can be explained if the effect of pH below 3.9 is to increase the frictional coefficient of the dispersed protein. The mobility would be reduced if this effect were greater than the effect of the increased charge. No experimental evidence can be advanced to support this suggestion.

Above the isoelectric range, mobilities were determined in buffers from just below pH 9 to pH 11. The plotted results yield a straight line with lower mobilities (approximately 7×10^{-5} cm²/volt/second) at the higher pH values. All of the buffers used were glycine – sodium hydroxide buffers with a constant concentration of glycine (0.05 *M*) and variable amounts of sodium hydroxide. The slope of the line is the opposite of that expected on the basis of the effect of pH on charge alone. The experimental results available do not permit an explanation.

There were no detectable species differences in the behavior of the globulins so far as mobility is concerned.

Discussion

The results presented in the preceding section strongly suggest that the crystalline globulin from the seeds of each of the four species of cucurbit studied is not a single protein. This conclusion is at variance with that reached as a result of sedimentation studies on the same material (1). If there are two or more proteins making up the crystals, it must be concluded that they are quite similar and probably vary only slightly in amino acid composition. Such similar proteins would not separate during sedimentation but might separate during electrophoresis analyses at critical pH levels. In this respect, therefore, Danielson's suggestion (9) that the earlier results (2) fit a two-component system has merit.

The globulin from each species of cucurbit appears to constitute an association–dissociation system sensitive to very small changes in the dispersion medium, particularly in the pH (1). The heavier components identifiable in sedimentation are reasonably explained as associations of the "monomer" which has an $S^0_{20,w}$ value of approximately 3.* The electrophoretic components cannot, however, be identified with those obtained in sedimentation, and this agrees with the much more limited results reported by Fuerst *et al.* (2). Sedimentation results do not show the very sharp differences that were obtained in electrophoretic analysis as a result of small changes in pH. The basic factors involved in the association and dissociation as measured by sedimentation seem therefore to be distinct from those resulting in the appearance of two distinct electrophoretic components in some preparations.

The results of the present study agree with those of the sedimentation investigations in showing that the properties of the globulins from the more closely related species, squash and pumpkin, are similar, but that these properties are measurably different from those of the globulins obtained from the more

*Since the paper presenting sedimentation results was published, a private communication from W. H. Cook has suggested that the aggregates yielding sedimentation constants of approximately 7 and 12 are probably not made up of two and four units of the monomer, but several times these numbers. No direct calculations can be made but an excellent case can be made for this view.

distantly related species, cucumber and watermelon. The latter two are generally similar under a variety of conditions but, considering all experimental results, differ more widely than do squash and pumpkin. These species differences are also in agreement with those based on amino acid analyses and reported by Smith and Greene (10). While no attempt has been made to relate the two types of study, it seems that differences in amino acid composition might account for the differences in sedimentation and electrophoretic behavior.

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EFFECT OF DIETARY OILS ON THE DEPLETION OF VITAMIN A[1]

T. K. MURRAY, J. L. BEARE, AND J. A. CAMPBELL

Abstract

The effect of dietary corn oil, olive oil, and rapeseed oil on the vitamin A content of liver and kidney during depletion of the vitamin was studied. Dietary oil had no influence on the rate at which liver stores of the vitamin declined in a 2-month period. Kidney stores of vitamin A rose throughout the depletion period but less markedly in the group fed rapeseed oil. The weight gain of vitamin A deficient rats was reduced somewhat earlier when rapeseed oil was fed but the survival time of rats fed corn oil or rapeseed oil was similar. The rats fed olive oil lived somewhat longer due to its small content of β-carotene. It was concluded that rapeseed oil had only a slight influence on vitamin A metabolism.

Introduction

Carroll reported (1) that erucic acid hastens the onset of vitamin A deficiency in rats deprived of fat-soluble vitamins and linoleic acid. On the other hand it was shown (2) that the storage of vitamin A and its disappearance from the liver was similar in rats fed corn oil and rapeseed oil, an oil high in erucic acid. Preliminary work in this laboratory indicated that during depletion of vitamin A in rats fed corn oil or rapeseed oil, more of the vitamin accumulated in the kidneys when corn oil was fed. The present experiment was designed to study further the effect of dietary oils on rate of vitamin A depletion.

Methods

One hundred and thirty weanling, male rats of an inbred Wistar strain were separated into 13 similar groups and randomized within blocks according to their initial body weights. At the beginning of the experiment one group was killed by decapitation, and the remaining animals supplied with the basal, vitamin A-free diet, previously described (2), to which was added 20% corn oil,[2] rapeseed oil,[3] or olive oil.[4] Food and water were supplied ad libitum; food consumptions and body weights were recorded twice weekly. Thirty animals receiving each diet were dosed orally with 1000 I.U. of vitamin A in 0.1 ml of cottonseed oil and subsequently killed at intervals of 2 days, 23 days, and 58 days. Livers and kidneys were analyzed for vitamin A by the antimony trichloride method of Ames, Risley, and Harris (3). The remaining rats, given no vitamin A, were reserved for a determination of their survival time.

Results and Discussion

The body weight of rats which had received the dose of vitamin A (Fig. 1) was significantly lower when the diet contained rapeseed oil instead of corn oil

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Contribution from the Food and Drug Laboratories, Department of National Health and Welfare, Ottawa, Ontario.
[2]Mazola.
[3]Golden rapeseed oil supplied by Dr. B. M. Craig and the Saskatchewan Wheat Pool.
[4]Pastene.

or olive oil. These weight differences were dependent upon the food consumption as was determined previously (4).

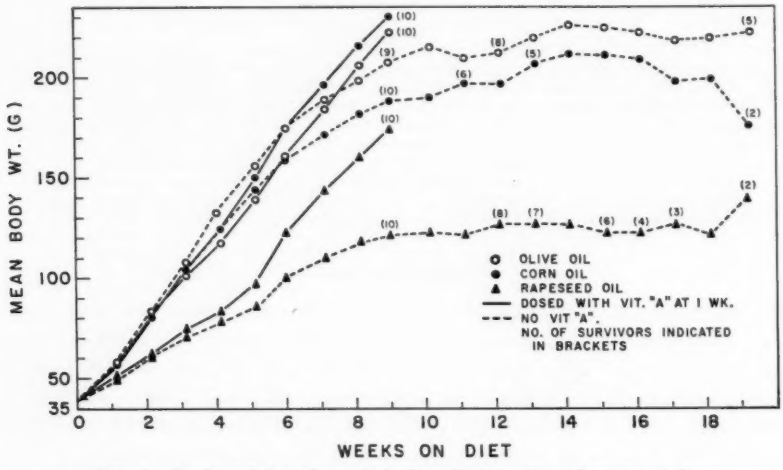

FIG. 1. Body weights of rats fed olive oil, corn oil, and rapeseed oil.

Differences in weight between rats which received the dose of vitamin A and those which did not became significant ($P = 0.05$) after 6 weeks when rapeseed oil was fed, after 7 weeks when corn oil was fed, and did not occur in 9 weeks when olive oil was fed. The earlier appearance of a weight plateau led Carroll (1) to conclude that erucic acid interfered with vitamin A utilization, and yet such a conclusion is not supported by survival data. Rats fed rapeseed oil and corn oil survived without vitamin A for approximately the same time. The better weight gains and survival times of rats fed olive oil and no vitamin A were attributed to a small amount of β-carotene detected by spectrophotometric examination of a nonsaponifiable fraction of this oil. This quantity, less than 25 I.U./100 g, was insufficient to affect liver stores or to change the vaginal smears of deficient rats. No β-carotene was found in corn or rapeseed oil.

The organ weights (Table I) of those animals killed at 9 weeks indicated that the absolute liver weight of rats fed rapeseed oil was significantly less than that of animals fed the other oils. When adjustment was made for body weight by covariance analysis, however, the liver was significantly heavier in animals fed rapeseed oil. The differences in kidney and testes weights were attributed to differences in body weight. As found previously with Wistar rats (2) the adrenal weights were not influenced by dietary oil.

Initially the stores of vitamin A in the liver and kidneys were 92 ± 4 and 1.5 ± 0.1 I.U. respectively. During the course of vitamin A depletion following the single dose, the total content of vitamin A in the liver (Fig. 2) was similar for each dietary oil, a finding which confirms a previous study (2) but does not support Carroll's contention (1) that erucic acid increases the requirement for vitamin A. As the liver stores of vitamin A declined, the relatively small

TABLE I

Body and organ weights of rats fed olive oil (OO), corn oil (CO), or rapeseed oil (RSO) for 9 weeks. Organ weights adjusted for body weights are shown in parentheses

Dietary oil	Body wt., g	Liver wt., g	Kidney wt., g/pr	Testes wt., g/pr	Adrenal wt., mg/pr
OO	223 ± 4*	8.04 ± .28 (7.45)	1.58 ± .04 (1.50)	2.38 ± .04 (2.30)	27.2 ± 0.9
CO	231 ± 12	8.24 ± .57 (7.29)	1.60 ± .07 (1.47)	2.40 ± .07 (2.27)	27.8 ± 0.9
RSO	174 ± 4	6.79† ± .32 (8.33‡)	1.31 ± .04 (1.52)	1.98 ± .08 (2.19)	28.0 ± 1.0

*Mean ± standard error.
†Significantly different at $P = 0.05$.
‡Significantly different at $P = 0.01$.

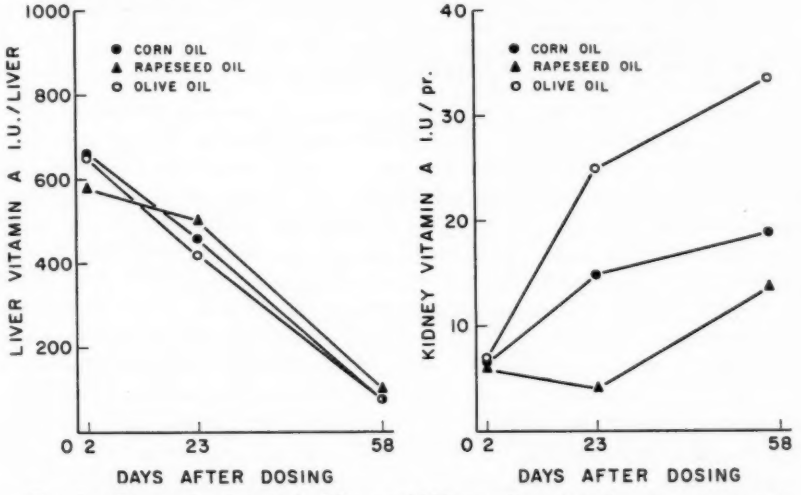

FIG. 2. Changes in vitamin A of liver and kidneys of rats fed olive oil, corn oil, and rapeseed oil following a single dose of the vitamin.

amount in the kidneys increased, a phenomenon observed by Johnson and Baumann (5) and Booth (6). The most interesting aspect of the change in kidney vitamin A was that the extent and rate of increase were significantly influenced by the dietary oils. From 2 to 23 days after dosing, the kidney vitamin A increased significantly when olive oil and corn oil, but not rapeseed oil, were fed, and the level became significantly different for each oil. Thereafter the kidney vitamin A increased with each dietary oil, and at the 58-day interval was not significantly different for rats receiving corn oil and rapeseed oil. The reduced transfer of vitamin A from liver to kidneys caused by rapeseed oil is in agreement with the observation of Johnson and Baumann (5) that suppression of growth by any means has this effect. The high level of kidney vitamin A obtained in rats receiving olive oil might be partly related to its small content of β-carotene. Very low levels of dietary vitamin A are known to affect kidney rather than liver stores (7).

The effect of rapeseed oil on the weight gain of deficient rats and on the vitamin A content of the kidney indicated only a very slight influence of this oil on vitamin A utilization. Rapeseed oil had no measurable effect on liver stores of vitamin A nor on the survival time of animals deficient in this vitamin.

Acknowledgments

The authors are grateful to Mr. C. Desloges for his care of the animals.

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THE INDUCTION OF METHAEMOGLOBINEMIA AS AN ADJUNCT TO THERAPY FOR TABUN POISONING[1]

I. W. COLEMAN, P. E. LITTLE, AND G. A. GRANT

Abstract

The protective and therapeutic effectiveness of *p*-aminopropiophenone (PAPP) against tabun poisoning in the rat has been examined. The relationship between dosage of PAPP and percentage of methaemoglobin in the blood of the rat has been determined.

Treatment of rats with PAPP both alone and in conjunction with atropine gave no evidence of protection against tabun poisoning. In combination with oxime treatment, PAPP improved protection against the use of monoisonitrosoacetone (MINA) by a factor of 4, but was less effective with diacetylmonoxime (DAM) and N-methylpyridinium-2-aldoxime methanesulphonate (P-2-S). The highest net protection from oxime and PAPP therapy was obtained with two members of the bisquaternary pyridinium-4-aldoxime alkanes, one member of which in the presence of PAPP protected rats from 23 LD_{50}'s of tabun.

Introduction

Treatment of poisoning by organophosphorous compounds currently employs three basic means to combat the effects of cholinesterase inhibition: atropine to reduce the muscarinic effect of accumulated acetylcholine; cholinesterase reactivators, usually oximes, to restore cholinesterase activity; and artificial ventilation if respiratory depression is severe (1, 2). Experimental evaluation of oxime and atropine effectiveness has shown that pyridine-2-aldoxime methiodide (P-2-AM), monoisonitrosoacetone (MINA), or diacetylmonoxime (DAM) are highly effective therapeutic drugs against Sarin, TEPP, DFP,* and other anticholinesterase agents (3, 4, 5, 6, 7). However, poisoning from dimethylamidoethoxyphosphoryl cyanide (Tabun) is notably resistant to prophylaxis or therapy from oximes and atropine. In this laboratory N-methylpyridinium-2-aldoxime methanesulphonate (P-2-S), an effective oxime for sarin poisoning when combined with atropine (8), has been found to give mice and rats protection from only 1.2 to 1.5 LD_{50} of tabun (9). The resistance of tabun to existing treatment procedures prompted the hypothesis that part of the failure of oxime and atropine therapy could be assigned to either the failure of oximes to produce effective reactivation of inhibited cholinesterase in vivo or to the effects of cyanide ion released in the reaction of tabun with cholinesterase (10). The latter hypothesis has been examined in the following study by the method of inducing methaemoglobinemia. Cyanide reacts with methaemoglobin to form cyanmethaemoglobin and in this combination is physiologically inert. Many agents have been used to induce methaemoglobinemia. Cox and Wendel (11) list 16 agents including acetanilide, *o*-amino-

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Contribution from the Defence Research Kingston Laboratory, Kingston, Ontario, of the Defence Research Board of Canada.

*The following common terms are used: Sarin, isopropylmethylphosphonofluoridate; T.E.P.P., tetraethylpyrophosphate; tabun, dimethylamidoethoxyphosphoryl cyanide; DFP, diisopropylfluorophosphate. The term "prophylactic" is restricted in meaning to the administration of a drug before exposure to an anticholinesterase agent. "Therapeutic" administration refers only to administration after exposure.

phenol, *o*- and *p*-nitrophenol. The agent chosen for this work was *p*-aminopropiophenone. Jandorf *et al.* (12) have demonstrated that treatment of dogs with PAPP significantly decreased the toxicity of cyanide and cyanogen chloride.

Experimental

The Toxicity of p-Aminopropiophenone

The toxicity of PAPP was determined, for the intraperitoneal route only, on male rats weighing 125 ± 10 g. Because of the low solubility of PAPP in aqueous or non-toxic organic solvents, the material for injection was prepared by extended grinding of PAPP in corn oil until a stable suspension was produced. The results of this assay indicate that the LD_{50} value of PAPP administered intraperitoneally lies between 525 and 575 mg/kg. This value was somewhat unexpected in view of the obvious cyanosis and respiratory difficulty exhibited by the animals at doses of 100 and 200 mg/kg. At values between 200 and 500 mg/kg the animals demonstrated gross cyanosis and were in a state of collapse from which they recovered. The recovery from what must have been a profound methaemoglobinemia likely reflects the high capacity of the rat to reduce methaemoglobin to normal haemoglobin. No information on the rate of reduction of methaemoglobin in the rat could be found in the literature, but Cox and Wendel (11) report the rate of reduction in dogs to be 11.3% per hour. From this study the rat would appear to demonstrate an even higher rate of reduction. Further evidence of the greater reductive capacity of the rat over the dog can be found in the difference in the toxicity of PAPP to the dog and rat. The LD_{50} of PAPP in the dog is given by the Handbook of Toxicology (13) as 7.5 ± 0.89 mg/kg. The LD_{50} of PAPP for the mouse is given in the same source as 223 ± 17 mg/kg. The mouse likely exhibits an intermediate capacity between the dog and rat to reduce methaemoglobin.

The lowest dosage tried in this study which gave an observable cyanosis, indicating a significant increase in methaemoglobin concentration, was 100 mg/kg. This dosage was chosen for therapeutic and prophylactic trials. However, a preliminary experiment to determine the relationship between the concentration of methaemoglobin produced and dose of PAPP administered was performed, in order to ensure that the production of methaemoglobin by this dose of PAPP was adequate for the detoxification of cyanide, in view of what must be a high rate of reduction of methaemoglobin in the rat.

Percentage Production of Methaemoglobin by Varying Doses of PAPP

p-Aminopropiophenone was prepared as before in a corn oil. Doses ranging between 50 and 150 mg/kg were given to each of three male rats at each dose with the agent administered intraperitoneally. The animals were sacrificed after 30 minutes by decapitation and the blood was collected using sodium heparinate (solid) as the anticoagulant. Percentage methaemoglobin was determined by the method of Michel and Harris (14) by measuring the absorption of the diluted blood sample at 634 mμ in a spectrophotometer before and after the addition of sodium cyanide. The results shown in Table I indicate that the methaemoglobinemia 30 minutes after the intraperitoneal injection

of PAPP is still appreciable. At a dose of 100 mg/kg the rat showed a mean methaemoglobin of 12.9% of the total haemoglobin.

TABLE I

Variation in methaemoglobin concentration of rats given *p*-aminopropiophenone intraperitoneally

Dose PAPP,* mg/kg	% methaemoglobin	Methaemoglobin, mean %
50	8.2, 11.3, 13.5	11.0
75	8.2, 11.3, 16.8	12.1
100	12.2, 13.0, 13.5	12.9
125	16.9, 22.8, 22.0	20.5
150	24.6, 24.6, 21.4	23.5

*Three animals were used at each dose of PAPP.

Prophylactic and Therapeutic Trial of PAPP against Tabun Poisoning

p-Aminopropiophenone was prepared as before in corn oil. The tabun sample used was dissolved in propylene glycol and on a separate toxicity trial demonstrated an LD_{50} of 0.210 mg/kg to the rat when administered subcutaneously. The animals used in the trial were healthy male rats weighing between 200 and 230 g. Atropine sulphate was dissolved in saline and administered intramuscularly at a dose of 12.5 mg/kg. In the prophylactic trials PAPP was given 15 minutes before tabun; in therapeutic trials it was administered 30 seconds after tabun.

TABLE II

Protection against tabun in male rats by *p*-aminopropiophenone

Group	No.	PAPP,* 100 mg/kg	Tabun,† 1 LD_{50}, 0.210 mg/kg	Atropine,‡ 12.5 mg/kg	Mortality
A	10	15 minutes before tabun	1.0 LD_{50}	None	10/10
B	10	30 seconds after tabun	1.0 LD_{50}	None	10/10
C	10	15 minutes before tabun	1.0 LD_{50}	14 minutes before tabun	9/10
D	10	15 minutes before tabun	1.0 LD_{50}	2 minutes before tabun	8/10
E	10	15 minutes before tabun	1.0 LD_{50}	30 seconds before tabun	9/10

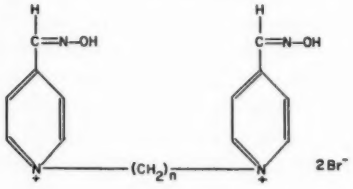
*PAPP administered in corn oil, intraperitoneal route.
†Tabun administered in propylene glycol by subcutaneous route.
‡Atropine sulphate in saline, by intramuscular route.

The results are shown in Table II. In none of the trials was any evidence obtained to indicate that PAPP, either alone or in conjunction with atropine, could protect or cure rats given one LD_{50} dose of tabun. Indeed the fact that in the total group of 50 rats used only four survived suggests that treatment with PAPP enhances the toxicity of tabun, since on one LD_{50} application, the expected survival would be nearer 25 animals. This result was not unexpected. Since the mode of death with this cholinesterase inhibitor is one of respiratory failure, it is not surprising that an agent such as PAPP, by increasing the degree

of anoxia through the removal of at least 12% of circulating haemoglobin as methaemoglobin, would impose a further respiratory strain on animals poisoned with tabun and have the effect of enhancing the toxicity of this agent.

Therapeutic Effectiveness of PAPP against Tabun Poisoning in Conjunction with Oximes and Atropine

It was apparent that the induction of methaemoglobinemia was of no value in protecting rats from tabun poisoning either alone or in the presence of atropine. In the following work, the treatment was extended to include the following oximes: monoisonitrosoacetone (MINA), diacetylmonoxime (DAM), P-2-S, and two members of a new group of oximes. These bisquaternary pyridinium-4-aldoxime alkanes have been shown (15, 16, 17) to be both potent reactivators of cholinesterase inhibitors in vitro and in vivo and to be effective in the therapy of poisoning by sarin and TEPP. There has been no published evidence, however, of the effectiveness of compounds 1 or 2 in tabun poisoning.



Compound 1
$n = 2$
Compound 2
$n = 3$

PAPP was administered intraperitoneally 15 minutes before injection of tabun at 100 mg/kg using corn oil as vehicle. Oximes were administered intramuscularly at doses of 50 mg/kg ½ minute after exposure to tabun with atropine sulphate (12.5 mg/kg im.) given 1 minute after the anticholinesterase agent. Rats of both sexes weighing between 175–200 g were exposed to doses of tabun graded so that the toxicity of tabun in treated animals could be determined. A minimum of six rats were used at each dose level. From these data, the Protective Index of the treatment was determined.

$$\text{Protective Index (P.I.)} = \frac{\text{Toxicity tabun in treated animals}}{\text{Toxicity tabun in untreated controls}} - 1.$$

All toxicity measurements are made on the LD_{50} calculated from the graphic procedure of Litchfield and Wilcoxon (18).

The results of the tests are shown in Table III. When PAPP was not given, MINA, DAM, and P-2-S produced no effective protection to tabun in the rat when used in conjunction with atropine. PAPP treatment raised the protection with each of these oximes to 4.0, 2.0, and 2.0 respectively. The bisquaternary pyridinium alkane oximes both produced significant protection to tabun when used with atropine. Compound 1 had a protective index of 3.0 and compound 2 the surprisingly high value of 19.0. Both oximes were increased in effectiveness by the presence of methaemoglobin from PAPP treatment such that the protective indices from compound 1 and compound 2 were increased to 5 and 23 respectively.

TABLE III

Protection against tabun poisoning afforded rats by intramuscular oximes and atropine in the presence of methaemoglobinemia induced by *p*-aminopropiophenone

Oximes used	Protective Index	
	Oxime* + atropine† without methaemoglobinemia	Oxime + atropine + PAPP,‡ methaemoglobinemia approx. 12%
MINA	0	4.0
DAM	0	2.0
P-2-S	0.2	2.0
Compound 1	3.0	5.0
Compound 2	19.0	23.0

*Oximes given intramuscularly, 0.5 minute after tabun at 50.0 mg/kg.
†Atropine administered intramuscularly, 1.0 minute after tabun at 12.5 mg/kg.
‡PAPP given intraperitoneally 15 minutes before tabun at 100 mg/kg.

These results clearly indicate that the induction of methaemoglobinemia in rats effectively increased the level of protection offered by all the oximes examined. Thus, although the treatment with PAPP is of no value in protection or therapy to tabun poisoning either alone or in the presence of atropine, it is of value when used in conjunction with oxime cholinesterase reactivating agents. The greatest increase with PAPP was found in combination with MINA. Undoubtedly this enhanced protection is partially due to the mode of action of MINA, which produces cyanide in the process of reactivation of inhibited cholinesterase or by direct reaction with organophosphorous cholinesterase inhibitors (19). This toxic action of MINA severely limits its use as a therapeutic agent. With PAPP treatment, the resultant methaemoglobinemia evidently removes the cyanide formed either by MINA or, in this case, by MINA and tabun, allowing the effectiveness of the oxime to be shown.

There is no evidence that the other oximes examined here release CN in the body by a reaction similar to that of MINA so that the effectiveness of PAPP under these conditions must be attributed to the detoxification of cyanide released from tabun. The net effect of this cyanide detoxification is not great but in the case of MINA, DAM, and P-2-S, it is sufficient to demonstrate that the cyanide released from tabun is an important factor in death from tabun poisoning and that removal of this cyanide permits the reactivation potencies of these oximes to be demonstrated. The bisquaternary oximes are the most potent reactivators of tabun-inhibited cholinesterase yet found (20). It is thus not surprising that these oximes provide a high protection to tabun poisoning. However, even when the order of protection to tabun was increased to 19 by the use of compound 2, PAPP treatment enhanced this value still further to a value of 23. With all the oximes except MINA, which is clearly in a category by itself due to the cyanide it produces during reactivation, the increase which can be attributed to PAPP is uniformly between two and three LD_{50}'s of tabun, although no reason for this limitation can currently be assigned.

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LOSS OF WEIGHT AND LIPID SHIFTS IN ORGANS OF THE DIGESTIVE TRACT OF TUMOR-BEARING RATS IN RELATION TO TUMOR SIZE[1]

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Abstract

The objective of this project was to find to what extent loss of weight in the digestive tract might affect tumor size and age in albino rats bearing Walker carcinosarcoma 256. Wet weight, dry weight, and water content were measured upon tongue, esophagus, cardiac stomach, pyloric stomach, jejunum, ileum, cecum, colon, and residual carcass (minus tumor). The animals bore tumors weighing 12 ± 6 (mean $\pm$ S.D.) % of host (minus tumor) weight after 18 ± 6 days of tumor growth (group I), 38 ± 12% after 24 ± 6 days (group II), and 93 ± 26% after 29 ± 5 days (group III) and controls were twins of the same sex. There were few significant changes in the animals of group I. In group II, there was loss of dry weight in all organs except pyloric stomach, losses being percentagewise the same as in residual carcass. Loss of dry weight of jejunum and ileum was less in rats of group III than in those of group II. In group III, loss of weight in other organs tended to be less than in residual carcass. Loss of dry weight in residual carcass was not significantly greater in the animals of group III than in those of group II. Water levels were increased in all organs of rats in groups II and III. This evidence indicates that rats of group III may have lived longer after tumor implantation, lost no more carcass weight, and bore larger tumors because they had lost weight in the small bowel at a lesser rate than had the rats of group II.

Further studies revealed that lipid shifts were in general less marked in organs which had lost the least weight. In animals bearing large tumors, percentage loss of neutral fat was less in most organs of the digestive tract than in the residual carcass. Increases in the levels of cholesterol and phospholipid were less in pyloric stomach and small bowel than in other organs of the digestive tract. Shifts in the amount of nonlipid dry weight and in levels of lipids and water were in general less in pyloric stomach and small bowel than in other organs of the body. In these respects, pyloric stomach and small bowel resembled brain, heart, and lung. It is suggested that resistance of pyloric stomach and small bowel to the cachectic influence of the tumor may be a factor determining tumor size and length of survival of the host.

Walker carcinosarcoma 256 is able to grow to a very considerable size. Some rats die when the weight of the grafted tumor equals one-fifth to two-thirds the weight of the host (minus tumor). Others survive until tumor weight approaches or exceeds host weight. Bloor and Haven (1) have suggested that death occurs when there is not enough intestinal tissue left in the host to support life and growth. They had found a marked loss of weight in the intestines of their tumor-bearing rats.

If weight of intestinal tissue is a factor limiting survival and tumor size, then rats which live longest and have the largest tumors should lose intestinal weight at the slowest rate. This should be particularly evident in organs of the digestive tract, such as pyloric stomach and small bowel, which are primarily concerned with digestion of food. It would be expected to be less evident in tongue, esophagus, cardiac stomach, cecum, and colon. It would be expected to be reflected in host weight to the extent that host participates with tumor in the benefits of a decreased loss of intestinal weight.

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Contribution from the Department of Pharmacology, Queen's University, Kingston, Ontario, Canada. This project was assisted by grants from Parke, Davis and Company and from the National Cancer Institute of Canada.

To investigate these possibilities, loss of weight was measured in the organs of the digestive tract and in host (minus tumor) carcass in albino rats bearing Walker carcinosarcomata 256 of three size ranges. Group I bore small tumors ranging from 1 to 19% of host weight. Group II had medium-sized tumors weighing 20 to 59% of host weight. Group III carried large tumors measuring 60 to 150% of host weight. Measurements were made before pre-mortem cachexia had set in.

Accompanying loss of dry weight in most organs of tumor-bearing rats, there occurs loss of neutral or storage fat. Loss of cholesterol and phospholipid is generally less extensive or may not even occur, so that levels of these lipids, per unit nonlipid dry weight, may actually increase during tumor growth (2).

Assuming that pyloric stomach, jejunum, and possibly other areas of the digestive tract are able to resist these effects of tumor growth to an extent greater than that of most other organs of the host, one might anticipate that there would be found in these areas of the digestive tract relatively minor changes in the levels of neutral fat, cholesterol, and phospholipid. Further, it might be expected that shifts in the level of these lipids would be least extensive in animals surviving longest and bearing the largest tumors.

Previously published data are available for duodenum (2) and combined small and large bowel (1) in rats bearing relatively large Walker tumors. In these studies, the mean levels of phospholipid were found significantly elevated, the mean levels of cholesterol unchanged, and the mean levels of neutral fat lowered.

Method

Twin albino rats were used. The animals were of a Wistar strain which has been bred since 1937 in the animal quarters of this department. Shortly after weaning, one of each pair of twins was inoculated subcutaneously with Walker carcinosarcoma 256, and the remaining twin kept under identical conditions as a control. The animals were fed Purina fox chow checkers and water ad libitum. There were approximately equal numbers of male and female twins in each group.

The organs of the digestive tract noted below in Table I were dissected and immediately weighed, after the contents of the lumen of the gut were removed. The craniad linear half of the small bowel was termed jejunum and the caudad half ileum. The remaining residual carcass (minus tumor) was weighed, cut into small pieces, and reduced to a homogenate.

Aliquots of each organ were analyzed for water content, neutral fat, total and free cholesterol, and phospholipid, by methods previously described (2). Lipids were measured as g per 100 g nonlipid dry weight of tissue. From a measurement upon a tumor-bearing rat (X_t) was subtracted the corresponding measurement upon its control, non-tumor-bearing twin (X_c) to obtain a difference ($X_t - X_c$). The difference was expressed as a percentage change from the control value by calculating $((X_t - X_c)/X_c) \times 100$. This made it possible to compare percentagewise shifts from organ to organ, and from group to group, after Croxton (3).

Results

A summary of changes in wet weight of tumor-bearers is given in Table I for animals in each of the three groups and for all animals combined. Values in the control twins were insignificantly different from each other in the three groups;

TABLE I

Percentage changes in the wet weight of organs of the digestive tract in albino rats bearing Walker carcinosarcomata 256 of different weights*

Organ	Tumor wt.: % host (minus tumor) wt. 1 to 19 $N = 14$ Group I	20 to 59 $N = 22$ Group II	60 to 150 $N = 17$ Group III	All tumor-bearers $N = 53$
Tongue	−9.0±5.9	−14.1±5.1	−15.9±3.9	−13.3±2.9
Esophagus	−0.8±5.2	−12.9±2.8	− 5.2±3.4	− 7.1±2.3
Cardiac stomach	−4.0±7.4	−13.0±3.8	−19.5±3.1	−12.7±2.9
Pyloric stomach	−0.2±5.2	− 1.4±2.6	+10.0±4.1	+ 2.5±2.4
Jejunum	−6.4±8.6	− 8.2±4.1	+14.5±6.6	− 0.5±3.9
Ileum	−0.1±6.4	−16.8±4.4	− 0.8±6.7	− 7.2±2.9
Cecum	−5.6±6.3	−17.0±3.5	− 2.7±5.6	− 9.4±3.0
Colon	+3.6±5.1	−14.0±3.1	− 1.2±5.2	− 5.2±2.7
Residual carcass (minus tumor)	−4.2±4.6	−18.5±4.1	−18.2±3.5	−14.8±2.5

*Percentage changes were measured as $((X_t - X_c)/X_c) \times 100$ where X_t was the wet weight in the tumor-bearer, and X_c the wet weight in its control, non-tumor-bearing twin. The results are expressed as mean ± standard error.

TABLE II

The wet weight, dry weight, and water content of the organs of the digestive tract in the control, non-tumor-bearing, twin albino rats

Organ	Wet wt.: g (mean ± S.E.)	Dry wt.: g (mean ± S.E.)	Water content: % wet wt. (mean ± S.E.)
Tongue	0.583±0.014	0.125 ±0.0039	78.7±0.31
Esophagus	0.175±0.0038	0.0330±0.0014	81.0±0.58
Cardiac stomach	0.313±0.012	0.0662±0.0027	79.2±0.40
Pyloric stomach	0.783±0.022	0.166 ±0.0066	78.4±0.37
Jejunum	3.21 ±0.11	0.584 ±0.017	81.4±0.21
Ileum	2.97 ±0.10	0.532 ±0.016	82.2±0.26
Cecum	0.794±0.030	0.141 ±0.0064	82.6±0.36
Colon	1.49 ±0.032	0.284 ±0.0086	81.0±0.34
Residual carcass	156 ±6.9	49.4 ±2.02	68.5±0.32

values for all controls combined are summarized in Table II. The mean ± standard deviation age of the tumor in days was 18±6 in the first group, 24±6 in the second, and 29±5 in the third. The mean ± standard deviation weight of the tumor, measured as % host (minus tumor) weight, was 12±6 in group I, 38±12 in group II, and 93±26 in group III.

Changes in wet weight of the organs in animals of group I were low and insignificant (Table I). In rats of group II, there was a significant loss in wet weight of all organs except pyloric stomach. In rats of group III, there were significant losses in weight only in tongue, cardiac stomach, and residual carcass; pyloric stomach and jejunum actually weighed more than in the control twins, and the loss of weight in esophagus, ileum, cecum, and colon

was less than in residual carcass. In all tumor-bearers combined, loss of weight was less in all organs except tongue, cardiac stomach, and cecum than in residual carcass.

Loss of wet weight in animals of group III was the same as in animals of group II in the cases of residual carcass, tongue, esophagus, and cardiac stomach. In all other organs of the digestive tract, from pyloric stomach to colon, there had occurred less loss of wet weight in animals of group III than in animals of group II.

Data upon water content are summarized in Tables II and III. All mean water levels were increased significantly except occasionally in animals of group I. In general, the edema was less extensive in the organs of the digestive tract than in residual carcass. Edema was of the same degree in animals of group III as in animals of group II except in the case of pyloric stomach. In pyloric stomach, water levels were higher in animals of group III.

TABLE III

Percentage changes in the water content of the organs of the digestive tract in albino rats bearing Walker carcinosarcomata 256 of different weights*

	Tumor wt.: % host (minus tumor) wt.			
Organ	1 to 19 $N = 14$ Group I	20 to 59 $N = 22$ Group II	60 to 150 $N = 17$ Group III	All tumor-bearers $N = 53$
Tongue	+0.88±0.86	+1.59±0.59	+1.71±0.84	+1.44±0.43
Esophagus	+1.04±0.96	+4.42±2.18	+3.90±1.84	+3.36±1.14
Cardiac stomach	+1.66±1.02	+3.38±1.00	+3.11±0.78	+2.84±0.57
Pyloric stomach	+1.69±0.74	+1.30±0.59	+4.33±1.01	+2.38±0.48
Jejunum	+0.74±0.52	+2.20±0.54	+2.69±0.37	+1.97±0.31
Ileum	+1.19±0.47	+3.50±0.65	+3.55±0.60	+2.90±0.37
Cecum	+1.44±0.74	+2.80±0.77	+3.81±0.83	+2.76±0.48
Colon	+1.00±1.08	+3.95±0.85	+4.23±0.87	+3.26±0.57
Residual carcass (minus tumor)	+1.22±1.21	+4.88±0.55	+6.69±1.08	+4.49±0.60

*Percentage changes were measured as $((X_t - X_c)/X_c) \times 100$ where X_t was the water content in the tumor-bearer and X_c the water content in its control, non-tumor-bearing twin. Water content was measured as g per 100 g wet weight of tissue. The results are expressed as mean ± standard error.

TABLE IV

Percentage changes in the dry weight of organs of the digestive tract in albino rats bearing Walker carcinosarcomata 256 of different weights*

	Tumor wt.: % host (minus tumor) wt.			
Organ	1 to 19 $N = 14$ Group I	20 to 59 $N = 22$ Group II	60 to 150 $N = 17$ Group III	All tumor-bearers $N = 53$
Tongue	− 6.9±6.3	−20.4±4.6	−18.0±2.7	−16.1±2.8
Esophagus	− 3.8±6.4	−26.8±3.9	−14.9±7.4	−16.9±3.6
Cardiac stomach	− 8.4±9.2	−22.4±3.0	−28.4±3.1	−20.6±3.1
Pyloric stomach	− 6.6±6.4	− 4.9±3.3	− 6.7±5.5	− 5.9±2.8
Jejunum	− 1.5±7.4	−16.9±4.2	+ 1.3±6.1	− 7.0±3.4
Ileum	− 4.1±6.5	−30.5±4.0	−19.3±4.8	−19.9±3.2
Cecum	−11.1±5.6	−27.2±3.8	−18.2±5.8	−20.0±3.0
Colon	+ 0.9±7.4	−26.7±3.0	−17.0±6.2	−16.3±3.5
Residual carcass (minus tumor)	− 7.2±4.1	−26.1±3.8	−29.8±3.3	−22.3±2.5

*Percentage changes were measured as $((X_t - X_c)/X_c) \times 100$ where X_t was the dry weight in the tumor-bearer and X_c the dry weight in its control, non-tumor-bearing twin. The results are expressed as mean ± standard error.

Summaries of measurements upon dry weight are listed in Tables II and IV. There were no significant shifts in animals of group I. All organs except pyloric stomach lost dry weight in animals of group II. In animals of group III, there was no significant loss of dry weight in pyloric stomach and jejunum, and loss of dry weight in other parts of the digestive system tended to be less than in residual carcass.

Loss of dry weight in animals of group III was less than in animals of group II in the jejunum and ileum. In other organs of the digestive tract and in residual carcass, loss of dry weight was not significantly different in group III from loss in group II.

A summary of lipid levels in the control twins is given in Table V. There were no significant differences between controls of groups I, II, and III.

TABLE V

Levels of neutral fat, free cholesterol, and phospholipid in the organs of the digestive tract of control, non-tumor-bearing albino rats*

Organ	Neutral fat	Free cholesterol	Phospholipid
Tongue	2.01±0.50	0.812±0.033	5.99±0.28
Esophagus	8.19±1.11	0.992±0.061	3.15±0.15
Cardiac stomach	6.86±0.88	1.333±0.118	3.02±0.26
Pyloric stomach	10.96±1.03	1.321±0.051	6.24±0.34
Jejunum	6.44±0.72	1.233±0.079	6.64±0.29
Ileum	11.09±1.27	1.114±0.038	5.57±0.21
Cecum	9.30±1.71	1.087±0.041	4.85±0.41
Colon	12.52±2.39	1.121±0.039	5.45±0.33
Residual carcass	13.82±0.95	0.536±0.026	3.71±0.20

*Levels were measured as g per 100 g nonlipid dry weight of tissue. The results are expressed as mean ± standard error.

TABLE VI

Percentage changes in the level of neutral fat of organs of the digestive tract in albino rats bearing Walker carcinosarcomata 256 of different weights*

Organ	Tumor wt.: % host (minus tumor) wt.			
	1 to 19 N = 11 to 15 Group I	20 to 59 N = 19 to 28 Group II	60 to 150 N = 15 to 22 Group III	All tumor-bearers N = 46 to 65
Tongue	+14.2±22.2	+18.6±17.3	− 6.2±15.9	+ 9.5±10.7
Esophagus	−12.8±11.5	+ 3.0± 9.2	−14.2±11.1	− 8.8± 6.2
Cardiac stomach	−48.5±13.9	− 6.9±11.7	−41.3±25.4	−27.6±10.6
Pyloric stomach	−32.1±19.1	−60.0± 8.0	−35.0± 9.0	−43.6± 6.7
Jejunum	−24.1±18.3	−34.4± 9.6	−41.2± 8.8	−33.9± 7.0
Ileum	−27.2±20.3	−34.7±15.0	−44.7±12.3	−35.8± 9.3
Cecum	−32.1±15.4	−14.5±20.2	−33.9±17.5	−25.6±10.8
Colon	−12.7±20.6	− 4.1±20.7	−60.6±15.9	−25.5±11.8
Residual carcass (minus tumor)	−11.9±14.5	−38.6± 9.3	−68.8± 5.3	−42.7± 6.3

*Percentage changes were measured as $((X_t - X_c)/X_c) \times 100$, where X_t was the level of neutral fat in the tumor-bearer and X_c the level in its control, non-tumor-bearing twin. Levels of neutral fat were measured as g per 100 g nonlipid dry weight. The results are expressed as mean ± standard error.

Shifts in levels of neutral fat have been summarized in Table VI. In groups I and II, shifts in the intestinal organs were in general of the same order as in residual carcass. In group III, the fall in levels of neutral fat was percentage-wise less in most organs of the digestive tract than in residual carcass.

Loss of neutral fat in the residual carcass of animals in group III was greater than that in groups I and II. This was not so in any organ of the digestive tract except colon. In pyloric stomach, loss of neutral fat was actually less in group III than in group II.

Shifts in levels of free cholesterol have been summarized in Table VII. No significant shifts occurred in the animals of group I. In group II, the percentage increase in levels of free cholesterol in residual carcass was of the same order

TABLE VII

Percentage changes in the level of free cholesterol of organs of the digestive tract in albino rats bearing Walker carcinosarcomata 256 of different weights*

Organ	Tumor wt.: % host (minus tumor) wt.			
	1 to 19 N = 11 to 15 Group I	29 to 59 N = 19 to 28 Group II	60 to 150 N = 15 to 22 Group III	All tumor-bearers N = 46 to 65
Tongue	− 7.8± 8.3	+17.3± 5.0	+19.3± 7.9	+11.0±4.3
Esophagus	− 2.3± 9.0	+20.1± 6.5	+14.2± 7.9	+13.0±4.4
Cardiac stomach	+10.4±10.9	+20.5± 8.0	+25.1± 9.0	+19.4±5.3
Pyloric stomach	− 0.2± 4.8	− 4.6± 3.3	+16.0± 3.1	+ 3.2±2.5
Jejunum	−10.4± 5.8	− 2.8± 3.0	+ 2.1± 4.3	− 3.2±2.5
Ileum	− 2.8± 4.5	+ 6.1± 5.0	+ 1.4± 9.5	+ 2.3±3.9
Cecum	− 2.1± 4.7	+20.3±10.0	+20.5±12.1	+13.9±5.9
Colon	+ 2.5± 8.6	+ 9.1± 7.1	+ 3.4± 7.6	+ 5.4±4.5
Residual carcass (minus tumor)	− 6.5± 7.6	+15.9± 5.2	+25.0± 6.3	+13.7±4.0

*Percentage changes were measured as $((X_t - X_c)/X_c) \times 100$, where X_t was the level of free cholesterol in the tumor-bearer and X_c the level in its control, non-tumor-bearing twin. Levels of free cholesterol were measured as g per 100 g nonlipid dry weight. The results are expressed as mean ± standard error.

as that in all organs of the digestive tract except pyloric stomach, jejunum, and possibly ileum. In the latter organs, the shifts were less than in residual carcass. In group III shifts were less in jejunum, ileum, and colon than in residual carcass.

TABLE VIII

Percentage changes in the level of phospholipid of organs of the digestive tract in albino rats bearing Walker carcinosarcomata 256 of different weights*

Organ	Tumor wt.: % host (minus tumor) wt.			
	1 to 19 N = 11 to 15 Group I	20 to 59 N = 19 to 28 Group II	60 to 150 N = 15 to 22 Group III	All tumor-bearers N = 46 to 65
Tongue	+16.8± 9.2	+16.9± 7.5	+27.9±16.4	+20.4±6.7
Esophagus	+ 1.7± 7.9	+12.7± 8.7	+43.3±20.4	+20.4±8.3
Cardiac stomach	+46.0±17.8	+21.5±10.9	+30.5±20.0	+30.4±9.4
Pyloric stomach	+ 5.2± 5.7	+ 4.8± 8.4	+17.5±14.9	+ 9.0±6.3
Jejunum	+ 4.3±14.4	− 7.3± 5.7	+ 9.9± 6.5	+ 1.2±4.9
Ileum	+ 1.7± 7.8	+ 0.2± 5.4	− 6.7± 9.5	− 1.5±4.3
Cecum	− 1.9± 7.0	+12.8±14.7	+46.1±22.3	+18.8±9.7
Colon	+13.2±10.5	+21.5± 9.8	+ 4.6± 7.1	+13.6±5.5
Residual carcass (minus tumor)	+ 2.0± 6.4	− 1.9± 8.4	+ 9.7± 8.8	+ 3.0±5.0

*Percentage changes were measured as $((X_t - X_c)/X_c) \times 100$, where X_t was the level of phospholipid in the tumor-bearer and X_c the level in its control, non-tumor-bearing twin. Levels of phospholipid were measured as g per 100 g nonlipid dry weight. The results are expressed as mean ± standard error.

Shifts in the levels of total cholesterol were insignificantly different from those of free cholesterol.

Changes in the levels of phospholipid have been listed in Table VIII. In most instances, increases in the level of this lipid were also less marked in pyloric stomach, jejunum, ileum, and possibly colon than in other organs of the digestive tract in groups II and III, and in all animals combined. Phospholipid levels were not significantly affected in residual carcass. This was due to the inclusion of skin. Phospholipid levels are decreased in skin and increased in the remaining residual carcass of rats bearing the Walker tumor (4).

Sex of the host rat was not a factor affecting any percentage shifts.

Discussion

These experiments demonstrated that rats bearing a large Walker carcinosarcoma 256 after a long period of tumor growth (group III) had lost less dry weight of small bowel than had rats bearing a medium-sized tumor after a medium length of tumor growth (group II). It is possible that the tumor grew to a large size in group III because growth of the small bowel had been inhibited by tumor growth to a lesser degree than in group II. Edema of the small bowel was no greater, as estimated by increase in water levels, in rats of group III than in rats of group II. This could indicate that digestive function per gram of small bowel was affected to the same extent in animals of both groups II and III. Since there was more dry weight of small bowel in animals of group III, the total amount of digestive function may have been greater, thus permitting a greater growth of tumor.

There was evidence that the residual carcass of the host (minus tumor) also derived some benefit from the larger small intestine of animals in group III. Loss of dry weight in residual carcass was statistically the same in this group as in rats of group II. The rats of group III, however, had lived significantly longer after tumor implantation than had the rats of group II. If loss of dry weight in residual carcass had regressed linearly with age of tumor at the same rate as between group I and group II, the loss in group III would have been much greater than was actually found. The evidence may be taken to indicate that rats of group III lived longer and bore larger tumors because they had lost weight of small bowel at a lesser rate during tumor growth than had the rats of group II.

The results indicate further that as the Walker tumor is able to continue its growth there occurs a progressive loss of neutral fat in residual carcass but not in the digestive tract. In animals which had survived the longest and bore the largest tumors (group III) loss of neutral fat was appreciably less in the digestive tract than in the remaining carcass. The results suggest that these latter animals may have been able to survive longer and bear larger tumors because their intestinal tract was able to resist the cachectic influence of the tumor upon its storage fat.

Accompanying this loss of neutral fat, there occurred significant increases in the levels of free cholesterol and phospholipid in tongue, esophagus, cardiac stomach, cecum, and possibly colon. No significant change in the level of these

lipids occurred consistently in pyloric stomach, jejunum, and ileum. Increases were by no means consistently greater in animals bearing the largest tumors. These results suggest again that ability to resist the effects of tumor growth is contained particularly in pyloric stomach and small bowel.

It is not possible to compare these shifts in the digestive tract with corresponding shifts in other organs of albino rats bearing the Walker tumor because exactly corresponding figures have not been published for other organs. Mean shifts, expressed as $(\bar{X}_d/\bar{X}_c) \times 100$ where $\bar{X}_d$ is the mean of the differences $X_t - X_c$, have been published or may be calculated from published data (2, 4–13) upon the organs listed in Table IX in albino rats bearing tumors

TABLE IX

Mean changes* in nonlipid dry weight† and in levels‡ of lipids and water in the tissues of albino rats bearing Walker carcinosarcoma 256

Tissue	Nonlipid dry weight	Neutral fat	Free cholesterol	Phospho-lipid	Water
Mesentery	− 74	−47	+92	+106	+182
Thymus gland	− 55	−18	+65	+ 35	+ 34
Diaphragm	− 44	−40	+38	+ 17	+ 41
Skeletal muscle	− 44	−54	+27	− 7	+ 17
Skin	− 42	−62	+40	− 21	+ 14
Submaxillary salivary glands	− 33	−28	+29	+ 8	+ 16
Cervical lymph nodes	− 31	−38	+30	+ 6	+ 20
Trachea	− 27	−31	+23	+ 19	+ 33
Cecum	− 22	−33	+19	+ 15	+ 17
Cardiac stomach	− 20	−29	+11	+ 3	+ 9
Testicle	− 20	−18	+49	+ 12	− 1
Tongue	− 20	+ 5	+17	+ 12	+ 9
Total body	− 20	−66	+10	+ 8	+ 10
Ileum	− 17	−62	+ 1	− 6	+ 15
Esophagus	− 17	− 4	+22	+ 15	+ 15
Kidney	− 15	− 6	+14	+ 16	+ 17
Colon	− 13	−65	+ 2	+ 9	+ 13
Jejunum	− 11	−36	− 1	− 2	+ 11
Hair	− 10	+16	− 1	+ 2	+ 2
Brain	− 6	+61	+13	+ 6	+ 6
Lung	− 6	− 6	+15	+ 4	+ 17
Pyloric stomach	− 2	−55	+ 1	− 1	+ 8
Heart	+ 2	+ 8	+28	+ 15	+ 21
Liver	+ 8	+51	+57	+ 13	+ 29
Adrenal§	—	—	−26	—	—
Spleen	+180	+15	−27	− 5	+ 11

*Calculated as 100 $(\bar{X}_d/\bar{X}_c)$, where $\bar{X}_d$ is the mean of the differences $X_t - X_c$, X_t the value in a tumor-bearer, and X_c the value in its non-tumor-bearing twin.
†Measured as grams.
‡Measured as g per 100 g nonlipid dry weight.
§Estimated from the data of Begg (12) and Haven, Bloor, and Randall (13).

approximately equivalent in size to those in groups II and III above. Corresponding figures were calculated from values upon organs of the digestive tract and included in Table IX. It should be remembered, when comparing figures in Table IX with similar figures in Tables VI to VIII, that $(\bar{X}_d/\bar{X}_c) \times 100$ is not necessarily the same figure as the mean of $((X_t - X_c)/X_c) \times 100$.

These comparisons revealed that mean shifts in pyloric stomach and small bowel were in general less than those of most other organs of the body. In these respects, pyloric stomach and small bowel react somewhat like brain, lung, and

heart in being able to resist to a greater extent than other host organs the cachectic effects of the Walker tumor upon weight, water, and lipids.

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THE ENZYME 5-PHOSPHORYLRIBOSE PYROPHOSPHOKINASE IN FISH MUSCLE[1]

H. L. A. TARR

Abstract

The enzyme 5-phosphorylribose pyrophosphokinase was found in the muscles, and in certain of the organs, of marine teleost fish. It was considerably purified at between 0 and −1° C by a simple, rapid procedure which involved extraction of the muscle with water, precipitation of the active fraction at pH 5.5, re-solution of the precipitate in 0.1 *M* phosphate buffer pH 7.0, and removal of about half the inactive protein from the solution by treatment with alumina C_γ gel. The enzyme was found to be very unstable, purified preparations losing activity quite rapidly even when stored at less than −50° C. The requirement of the enzyme for ATP, R5P, Mg^{++}, and glutathione, and its pH–activity relationship, were investigated. The lithium salt of PRPP was prepared in good yield and in a comparatively high state of purity using the purified muscle enzyme from lingcod.

Introduction

Kornberg *et al.* (1) were the first to report the isolation of PRPP and they subsequently gave a more detailed account of its enzymic synthesis from ATP and R5P by a pigeon liver enzyme (Kornberg *et al.* (2)). In independent studies which were largely concerned with the biosynthesis of purines, Korn *et al.* (3) and Remy *et al.* (4) isolated the same compound and showed that the pyrophosphate group was in the α position. Tener and Khorana (5) achieved chemical synthesis of the compound as the lithium salt, though in rather poor yield. They produced unequivocal evidence that the position of the pyrophosphate group was α. Khorana *et al.* (6) showed that the enzymic synthesis of PRPP involved a direct transfer of the terminal pyrophosphate group of ATP to carbon 1 of R5P. The pivotal role which PRPP plays in biosynthesis of purine and pyrimidine nucleotides was reviewed by Carter (7) and more recently by Hartman and Buchanan (8). The last-named investigators described the complex series of enzymic processes which are involved in the *de novo* synthesis of inosinic acid from R5P and ATP via PRPP and other intermediates.

Materials and Methods

In preliminary studies a sample of lithium PRPP kindly supplied by Dr. Gordon Tener of the B.C. Research Council was used for reference. In the initial stages of the investigation the dried yeast used to prepare the "alcohol fraction" containing orotodine 5′-phosphate pyrophosphorylase and carboxylase enzymes used in the enzymic assay of PRPP (Lieberman *et al.* (9)) was that which had been used by Dr. A. Kornberg. This yeast, which was generously donated by Dr. Gobind Khorana of the B.C. Research Council, had been stored

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The following abbreviations are used: PRPP, 5-phosphoryl α-D-ribofuranose 1-pyrophosphate; R5P, D-ribose 5-phosphate; DR5P, 2-D-deoxyribose 5-phosphate; AMP, adenosine 5′ phosphate; ADP, adenosinediphosphate; ATP, adenosinetriphosphate; Tris, tris(hydroxymethyl)aminomethane; PRPPkinase, 5-phosphorylribose pyrophosphokinase.

frozen for several years and had lost much of its original activity. It was found subsequently that a water-washed, air-dried "starter" yeast, kindly supplied by Mr. J. Neilson of the Vancouver branch of Carling Breweries, Ltd., gave an enzyme preparation with activity practically identical with that described by Lieberman *et al.* (9). The dried yeast and the enzyme preparations made from it were stored over dry ice (less than −50° C) during the course of the work.

Barium deoxyribose 5-phosphate was prepared as described by Kornberg *et al.* (2). It was about 95% pure as determined by its content of deoxyribose and phosphorus and the fact that almost complete hydrolysis of the orthophosphate group occurred in 30 minutes in 1 *N* HCl at 100° C (Racker (10)). Barium ribose 5-phosphate was prepared by the method of Khym *et al.* (11). Aqueous solutions of these compounds were converted to the sodium salts before use by treatment with Dowex 50 × 8 Na^+ resin. Disodium ATP and ADP were purchased from the Pabst Laboratories, Milwaukee, Wisconsin. Reduced glutathione and D-ribose were obtained from the Nutritional Biochemical Corporation, Cleveland, Ohio, and AMP from Schwarz Laboratories, Mt. Vernon, New York. Aged alumina gel Cγ (40 mg dry weight per ml) was kindly donated by Dr. W. E. Razzell of the B.C. Research Council.

Ribose was determined by the Mejbaum (12) method with 40-minute heating and D-ribose as standard. Orthophosphate in the presence of labile phosphate was determined by the method of Lowry and Lopez (13) with the modification suggested by Bruemmer and O'Dell (14). Total phosphorus was determined by the Gomori (15) procedure after hydrolysis (Umbreit *et al.* (16)). Protein was determined by the Kingsley (17) procedure. PRPP was determined by the assay B, stage II, procedure of Kornberg *et al.* (2). With the cruder enzyme preparations, PRPP formation was stopped and protein removed before assay by immersing the glass tubes containing the reaction mixtures for 40 seconds in a boiling water bath, promptly chilling the solutions in a dry-ice–alcohol bath, and centrifuging them. In experiments with purified enzyme preparations it was found that direct assay of the extracts was usually satisfactory since PRPP formation was comparatively slow at 30° C.

Experimental

With fish obtained at sea the fisherman placed samples of freshly excised muscle in polyethylene bags and froze them in contact with dry ice. Live lingcod (*Ophiodon elongatus*) were supplied through the courtesy of Mr. Murray Newman, Curator of the Vancouver Aquarium. The fish were stunned, the muscle and organs promptly excised, frozen in liquid nitrogen, and, unless otherwise stated, stored at below −50° C in polyethylene bags.

In preliminary studies the distribution of PRPPkinase in different tissues or organs of fish was determined by blending a portion with 2 volumes of water at 0° C for about half a minute and centrifuging at 10,000 *g* for 5 minutes. The clear extracts were tested by adjusting them to between pH 7.4 and 7.5 and incubating 0.33 ml in a total volume of 1.0 ml with ATP and R5P exactly as in the assay B, stage I, procedure of Kornberg *et al.* (2). PRPP formation was normally linear for up to 6 hours at 30° C under these conditions. This assay

was adopted since it was the only one known which was available for studying PRPP formation. Toward the end of the investigation it was found that the ATP and GSH concentrations as specified in this procedure were somewhat suboptimal for the fish muscle enzyme, but it was felt that the differences did not warrant a complete reinvestigation using more optimal concentrations of these compounds.

The results of tests with a number of aqueous tissue extracts (Table I) indicated that, apart from spleen and milt, only the muscle showed considerable activity. The lability of the enzyme system in the muscle and crude extracts is

TABLE I

Formation of PRPP by aqueous extracts of fish tissues

Source*	Treatment	Protein (mg/ml) of extract	Specific activity† ($\times 10^3$)
Lingcod muscle[1]	Muscle stored 10 days at −25° C	9.7	15.6
Lingcod muscle[1]	Aqueous extract stored 3 days at −25° C	9.7	13.8
Lingcod muscle[2]	Fresh muscle stored 2 hours at 0° C	10.2	14.4
Lingcod muscle[2]	Frozen muscle stored 24 hours at −25° C	10.2	13.5
Lingcod muscle[2]	Frozen muscle stored 34 days at −25° C	10.2	12.3
Lingcod muscle[2]	Frozen muscle stored 42 days at −25° C	10.2	7.3
Lingcod muscle[2]	Aqueous extract freeze dried, redissolved	10.2	13.8
Lingcod muscle[3]	Fresh muscle stored 2 hours at 0° C	10.3	12.1
Lingcod muscle[3]	Aqueous extract stored 2 days at less than −50° C	10.3	11.6
Lingcod muscle[3]	Aqueous extract stored 2 days at −25° C	10.3	9.3
Lingcod muscle[3]	Aqueous extract stored 1 day at 1° C	10.3	7.8
Lingcod muscle[3]	Aqueous extract stored 4 days at 1° C	10.3	2.6
Lingcod muscle[3]	Aqueous extract adjusted to pH 9.0 and promptly readjusted to pH 7.1 at 0° C	10.3	8.1
Lingcod muscle[4]	Frozen at sea in dry ice	10.6	9.1
Lingcod muscle[5]	Frozen at sea in dry ice	10.3	8.5
Lingcod muscle[6]	Frozen at sea in dry ice	11.1	5.4
Coho salmon muscle	Frozen at sea in dry ice	9.8	8.3
Spring salmon muscle[1]	Frozen at sea in dry ice	10.0	9.0
Spring salmon muscle[2]	Frozen at sea in dry ice	10.4	7.3
Blue perch	Frozen at sea in dry ice	9.7	7.5
Lingcod spleen[3]	Stored 18 days at less than −50° C	33.0	3.0
Lingcod liver[3]	Stored 18 days at less than −50° C	40.0	0.22
Lingcod heart[3]	Stored 18 days at less than −50° C	33.5	0.36
Lingcod milt[3]	Stored 18 days at less than −50° C	8.0	2.7

*Numbers indicate different fish. All fish muscles or organs were excised from stunned fish and promptly frozen by immersion in liquid nitrogen unless otherwise specified.

†Micromoles of PRPP formed by 1 mg of protein in 1 hour at 30° C under the conditions of the standard assay (stage I).

apparent since the activity of preparations obtained by freezing the muscle in dry ice at sea was invariably lower than that obtained with muscle frozen promptly in liquid nitrogen. Also storage of the muscle, or of aqueous extracts prepared from it, at −25° C caused slow loss in activity, while in extracts held at 1° C inactivation was extremely rapid. Brief exposure to pH 9.0 at 0° C resulted in marked loss of activity. The crude muscle extracts lost little activity when partially concentrated by freeze-drying.

Preparation of Enzyme

The lability of the enzyme rather limited available purification techniques. Dialyzed ammonium sulphate precipitates made at pH 7.0 and 0° C possessed

no PRPPkinase activity. Likewise, adsorption of crude lyophilized active muscle extracts on diethylaminoethyl cellulose columns followed by gradient elution at low temperatures as used in previous work (Tarr (18)) yielded inactive protein fractions. The following rapid method resulted in considerable purification with little loss in the total activity. All steps were carried out at 0 to $-1°$ C as rapidly as possible.

Frozen lingcod muscle was shaved off, mixed well with an equal volume of water in centrifuge tubes, and centrifuged at 10,000 *g* for 10 minutes. The extraction was repeated and the pooled supernatant liquids filtered through a thin layer of glass wool and adjusted cautiously to pH 5.5 with 1 *N* acetic acid. The precipitate was collected by centrifuging 3 minutes at 10,000 *g*, the supernatant liquid drained off, and the precipitate dissolved in 0.1 *M* sodium phosphate buffer, pH 7.6, to give a total volume equal to about one-eighth of that of the original extract. This solution (pH about 7.0) was stored at less than $-50°$ C, since under these conditions it retained most of its original activity for 2 or 3 weeks. Prior to use, 1 volume of freshly washed alumina Cγ gel (40 mg solids per ml) was added to 4 volumes of the extract, and the mixture centrifuged 10 minutes at 10,000 *g*. This procedure removed about half the protein without significant loss of total activity. Attempts at further purification using calcium phosphate gel were not successful.

Table II gives the protocol of a typical purification which shows that the enzyme is concentrated about 30-fold on the basis of the original aqueous extract without serious loss of total activity. The muscle used had been stored

TABLE II

Preparation of 5-phosphorylribose pyrophosphokinase

Fraction	Volume (ml)	Protein (mg)	Total units*	Specific activity†
Aqueous extract of 20 g muscle	42.0	425	9.3	0.022
pH 5.5 precipitate	5.0	22.8	9.25	0.41
pH 5.5 supernatant	43.0	394	1.45	0.0036
Alumina Cγ gel supernatant	5.5	12	8.30	0.69

*Amount of enzyme forming 1 μmole of PRPP per hour at 30° C under the conditions of the standard assay (stage I) is one unit.
†Units per mg of protein.

4 months at less than $-50°$ C and had lost only a little of its original activity. On the assumption that the fish muscle contained 18% protein, purification would be about 250-fold assuming no loss of activity occurred in preparation of the aqueous extract. The specific activity of another Cγ alumina gel treated preparation was 1.0. On a protein basis the most active preparation had about 1/30 of the specific activity of the best preparations made from acetone-treated pigeon liver (Kornberg *et al.* (2)). Preparations treated with alumina Cγ gel were used soon after preparation, for even when stored at less than $-50°$ C they lost their activity in a few weeks.

pH *Activity*

Assay B, stage I, medium (subsequently referred to as the standard medium) of Kornberg *et al.* (2) was prepared without added enzyme, and portions of the

chilled solution were adjusted to the pH values indicated in Fig. 1 by addition of very small amounts of 0.025 *M* acetic acid or 0.1 *M* tris solution. Glass tubes (7.5 × 100 mm) containing 0.95 ml of buffered solution and 0.5 ml

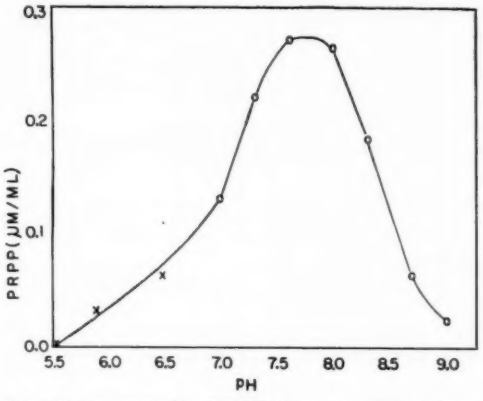

FIG. 1. The pH–activity curve for PRPPkinase. Acetate, ×——×; Tris buffers, ○——○.

(375 μg protein) of purified PRPPkinase were incubated 3 hours at 30°. PRPP formation was approximately linear at all pH values studied for this period. The tubes were immersed 40 seconds in a boiling water bath, promptly chilled in a freezing mixture of dry ice and alcohol, and centrifuged to remove precipitated protein. PRPP was determined enzymically using 0.20-ml portions in the standard assay. The results (Fig. 1) show that the kinase has a fairly sharp optimum between pH 7.5 and 8.0, is inactive at pH 5.5, and almost inactive at pH 9.0.

Effect of R5P and ATP Concentrations on PRPP Formation

Tubes containing the constituents of the standard assay medium were prepared, the concentration of R5P being varied with the ATP concentration constant at 1.8×10^{-3} *M*, and the ATP concentration varied with the R5P concentration constant at 1.25×10^{-3} *M*. Each tube received purified enzyme (300 μg protein) in a total volume of 1 ml. The amount of PRPP present was determined after 3 hours at 30° C, using 0.2-ml aliquots. The results (Fig. 2) show that a concentration of about 5×10^{-4} *M* R5P is required for maximum activity, and that concentrations in excess of this and not exceeding 5×10^{-3} *M* do not affect PRPP formation. On the other hand, PRPP synthesis is reduced when concentrations of ATP above about 2.5×10^{-3} *M* or below 1.5×10^{-3} *M* are used. No PRPP synthesis occurred when DR5P replaced R5P in the standard method, even when the concentration was increased to 5.0 μmoles/ml.

Effect of Mg^{++} *Glutathione and* F^-

The concentrations of reduced glutathione, or of Mg^{++} (as $MgCl_2$) were varied in the standard assay medium, each tube receiving 370 μg of purified enzyme protein. The PRPP concentration was determined as usual after

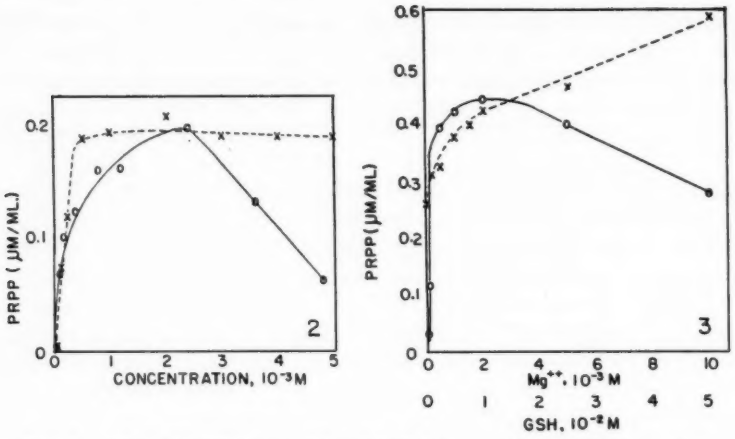

FIG. 2. Effect of ATP (○——○) and R5P (×- - - ×) on PRPP formation.
FIG. 3. Effect of Mg^{++} (○——○) and GSH (×- - - ×) on PRPP formation.

2 hours at 30° C. The results (Fig. 3) show that the enzyme has an absolute requirement for Mg^{++} and that the optimum concentration of this cation is about 2×10^{-3} *M*. PRPP formation proceeded in the absence of added glutathione, but its addition occasioned an increase in the amount of PRPP formed which was maximal at the highest concentration of glutathione studied (5×10^{-2} *M*). Kornberg *et al.* (2) did not indicate whether the concentration of GSH used in their assay was optimal.

In the absence of KF only about half as much PRPP was formed as when 0.01 to 0.10 *M* KF was added to the standard assay medium. However, in 0.20 *M* concentration, fluoride was inhibitory.

Balanced Studies of the Reaction

Kornberg *et al.* (2) showed that, under the conditions of the standard assay where the initial concentrations of ATP and R5P were 1.2×10^{3} *M* and 2.5×10^{-3} *M* respectively, about 90% of the ATP was converted to PRPP and AMP. In the present work, with the enzyme preparations used it has not been possible to achieve this condition. Thus, under the conditions of the standard assay (225 μg/ml enzyme protein), and commencing the reaction with 0.25, 0.50, and 1.0 μmole per ml each of R5P and ATP, only approximately 80%, 60%, and 40% respectively of the reactants could be accounted for as PRPP (Fig. 4). Even when three times the concentration of enzyme was employed with 1 μmole/ml of reactants, only 58% could be accounted for as PRPP after 5 hours, there being no further increase up to 7 hours. A slow linear decrease in PRPP concentration occurs in the complete assay medium when heat-inactivated enzyme is used, presumably due to formation of 5-phosphorylribose 1,2-cyclic phosphate. This is by no means as marked as that which occurs when PRPP and AMP are incubated with the active enzyme preparation (Fig. 4). The prompt decrease in PRPP which occurs under the above conditions has

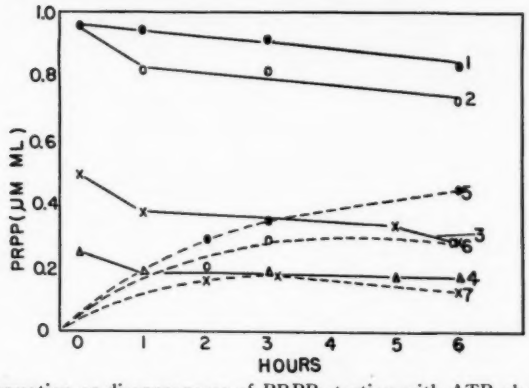

FIG. 4. Formation or disappearance of PRPP starting with ATP plus R5P or with PRPP plus AMP. Curve 1: 1 μmole each of PRPP plus AMP, heated enzyme control. Curves 2, 3, and 4: 1.0, 0.5, and 0.25 μmole/ml each of PRPP plus AMP. Curves 5, 6, and 7: 1.0, 0.5, and 0.25 μmole/ml each of R5P plus ATP. Enzyme concentration 225 μg protein per ml.

not yet been explained, but it could be at least partially accounted for by the possible presence in the enzyme preparation used of purine or pyrimidine pyrophorylase enzymes. Such enzymes could cause reaction of PRPP with small amounts of these bases, which may occur in the enzyme preparation, to form nucleotides (Kornberg *et al.* (19)).

When mixtures containing 0.5 μmoles/ml of AMP and PRPP were incubated 2 hours at 30° with purified enzyme under the above conditions, reduced to a very small volume by freeze-drying, and the concentrates submitted to descending chromatography using an isobutyric acid solvent system (Zetterström and Ljunggren (20)), no R5P or ATP could be demonstrated. With paper electrophoresis of the concentrates using Whatman No. 3 paper and 0.05 *M* sodium citrate buffer, pH 3.5 (30 v/cm/1.5 hours) (Smith (21)), a system which occasions excellent separation of ATP, ADP, and AMP, no trace of ATP was found when the appropriate area of paper was eluted and the solution examined spectroscopically at 258 mμ. This indicates that the reaction is almost certainly irreversible. A large proportion of the AMP had disappeared and a zone which corresponded with IMP had appeared in both systems, indicating the presence of a strong AMP deaminase in the enzyme preparation.

Preparation of PRPP

The general procedure of Khorana *et al.* (6) was followed. ATP, 1 mmole; R5P, 1 mmole; glutathione, 10 mmole; KF, 50 mmole; $MgCl_2$, 2 mmole; potassium phosphate buffer (pH 7.6), 20 mmole; and 50 ml of purified PRPP-kinase (188 mg protein) in a final volume of 1 liter were incubated at 30° C. After 4 hours, 430 μmoles of PRPP were present and the amount did not increase appreciably after a further 2 hours' incubation. The solution was chilled, treated with 50 g of Norit A acid-washed charcoal, which was sufficient to remove practically all substances causing absorption at 260 mμ, centrifuged at 3° C for 2 minutes at 10,000 *g*, and filtered by gravity through Whatman

No. 31 fluted filter paper. The charcoal treatment occasioned a considerable loss of PRPP since assays showed 328 μmoles were present at this stage.

All subsequent operations were carried out below 3° C. The solution was absorbed on a 20 × 5 (diam.) cm column of Dowex 1 × 10 (200–400 mesh) resin in the chloride form, the column washed with 1 liter of water, and elution carried out at 2 liters per hour, first with 9 liters of 0.1 *M* LiCl (pH about 6.3) to remove Pi and R5P and subsequently with 0.2 *M* LiCl. The first 360 ml of eluate was discarded, the PRPP (320 μmoles) being eluted in the next 2 liters. The clear solution was concentrated to 95 ml by freeze-drying, mixed with anhydrous methanol (38 ml) and acetone (380 ml), and the precipitated lithium salt of PRPP collected by centrifuging 2 minutes at 5000 *g*. The precipitate was suspended in 25 ml of a solution containing 1 part of methanol and 2 parts of acetone, centrifuged, resuspended in 50 ml of acetone, and the precipitate dried 5 minutes over P_2O_5 using a high vacuum pump to yield 160 mg of a white powder. This gave the following analysis, in micromoles per milligram, calculated for $C_5H_7O_{14}P_3Li_5.5H_2O$; M.W. 508.82 (Tener and Khorana (5)).

	Theoretical value	Found	% of theoretical
Total ribose	1.97	1.71	87
Total P	5.90	5.15	87
Inorganic P	0	0.063	1.07
PRPP (enzymic assay)	1.97	1.66	84.5
Ratio, organic P:ribose	3:1	3.05:1	
Organic P	5.90	5.087	86

Since 84.5% of the total ribose (87%) was accounted for as PRPP, not more than 2.5% of the ribose was present as the 1,2-cyclic ester of PRPP. The yield was 266 μmoles calculated from the enzyme assay, or 62% of the amount formed initially, most of the loss occurring during the charcoal treatment.

Discussion

Since the original work of Kornberg *et al.* (2) in which it was reported that extracts of acetone powders of livers of the chicken, rat, and mouse had very much lower PRPPkinase activities than did those of pigeon liver, the distribution of this enzyme in other mammalian tissues does not appear to have been studied. The specific activities of the best preparations of fish muscle PRPPkinase reported in the present work, though much lower than that of the most active pigeon liver preparations, are not very different from those reported by Kornberg *et al.* (2) for livers of animals other than that of the pigeon. So far as the writer is aware, the presence of this enzyme has not been reported in mammalian muscles.

The general properties of the enzyme from fish muscle so far as pH–activity relationship, magnesium requirement, optimum ATP, and R5P concentrations and inability of DR5P to replace R5P are rather similar to those reported for the pigeon liver enzyme. The enzyme was found to be present, and in about the same concentration, in the muscles of several marine teleost fish. The PRPPkinase activity of liver and heart extracts was very poor compared with muscle, while spleen extracts had lower activity.

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NUTRITION AND METABOLISM OF MARINE BACTERIA

IX. ION REQUIREMENTS FOR OBTAINING AND STABILIZING ISOCITRIC DEHYDROGENASE FROM A MARINE BACTERIUM[1]

ROBERT A. MACLEOD, AIKO HORI,[2] AND SYLVIA M. FOX

Abstract

When cells of a marine bacterium were washed and suspended in 0.05 *M* $MgCl_2$, no isocitric dehydrogenase could be demonstrated in the extract prepared, although all other tricarboxylic acid cycle enzymes had been shown to be present. Failure to detect the enzyme was traced to the presence of halide as the sole anion in the solution used to suspend the cells. Sulphate or phosphate salts gave rise to an active enzyme. These relations applied to extracts prepared by sonic treatment, alumina grinding, and lysis by versene and lysozyme. Evidence was obtained suggesting that halides fail to prevent the denaturation of the enzyme after its release from the cells. Tested under optimum conditions, the specific activity of isocitric dehydrogenase in the extract was found to be proportional to the ionic strength of the solution used to suspend the cells (maximum activity at 0.4 to 0.5 μ). In the presence of low concentrations of an anion giving rise to an active enzyme, halide salts were found capable of contributing to these ionic strength requirements. The addition of Mg^{++} to the extract in the presence of a suitable anion at least doubled the storage life of the enzyme at 4° C.

Introduction

In the course of an investigation to determine if enzymes of the tricarboxylic acid cycle were present in a marine bacterium (1, 2), cells of the organism were routinely washed and suspended in a solution of $MgCl_2$ for the preparation of extracts by sonic treatment. A solution of $MgCl_2$ was the medium of choice for washing the cells since Mg^{++} prevented cell lysis (3) and the enzymes in the extracts prepared were to be tested for their response to Na^+ and K^+. All of the enzymes of the tricarboxylic acid cycle could be shown to be present in the extracts except isocitric dehydrogenase. Since citrate-adapted whole cells of the organism had been found to oxidize citric acid with the accumulation of large amounts of α-ketoglutaric acid (4) it seemed likely that isocitric dehydrogenase was present in the intact cells. Conditions were therefore sought for the demonstration of its presence in the cell-free extract. These studies have indicated that rather specific inorganic requirements must be met before an active isocitric dehydrogenase can be demonstrated routinely and its activity maintained.

Experimental

The organism used is a species of either a *Pseudomonas* or a *Spirillum*, and has been designated B-16 in this and previous communications.

Conditions for carrying the cultures, growing the cells, and preparing cell suspensions have been described (2). After being harvested from the medium by

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Contribution from the Fisheries Research Board of Canada, Technological Station, Vancouver, B.C.

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[3]Abbreviations used: Tris, tris(hydroxymethyl)aminomethane; *G*, gravity; TPN, triphosphopyridine nucleotide; μ, ionic strength.

centrifugation, cells were resuspended in a volume of wash solution equal to half the volume of the original medium. The cells were then resuspended in and centrifuged from the appropriate salt solution twice more before final suspension. All manipulations were conducted at 4° C.

Cell-free extracts were prepared in three ways. Sonic extracts were obtained by treating cell suspensions for 5 minutes in a Raytheon 10-kc sonic oscillator. Extracts by alumina grinding were prepared by the method of McIlwain (5). A cell paste was mixed with two and one-half times its wet weight of levigated alumina and rubbed with a pestle in a chilled mortar until cell breakage occurred. Cell extracts by cell lysis were obtained by applying the procedure of Repaske (6) modified to provide conditions optimum for the lysis of this organism. The lysing system contained (in amounts/ml): Tris[3] buffer 6.25 μmoles, versene 4.3 μmoles (adjusted to pH 7), crystalline lysozyme (Worthington Biochemical Corp.) 0.05 mg, cell suspension to provide 10 mg dry weight of cells per ml of the lysing system. When an all-sulphate system was desired, Tris buffer was added as the sulphate salt and K_2SO_4 was added to bring the final ionic strength to 0.3. For an all-chloride system, buffer was added as the chloride salt and the ionic strength adjusted with KCl. The mixture was incubated for 30 minutes at room temperature and then made 0.05 *M* with respect to Mg^{++} using either $MgSO_4$ or $MgCl_2$, depending on the anion desired. Desoxyribonuclease was added at a level of 0.1 mg/ml and incubation allowed to proceed for a further 30 minutes.

All extracts were finally clarified by centrifuging for 30 minutes at 50,000 $\times$ *G* in a Spinco ultracentrifuge.

Both isocitric dehydrogenase and aconitase were measured spectrophotometrically, the former by following the reduction of TPN at 340 mμ (7), the latter by determining the rate of increase in optical density at 240 mμ (8), in both cases using *dl*-isocitrate as substrate. The compositions of the test systems used are included as footnotes to the tables.

A unit of enzyme activity was taken to be that amount of enzyme producing an optical density change of 0.001 per minute. Specific activity was calculated to be the number of enzyme units per mg of protein.

Protein was determined by the biuret procedure (9) using crystalline pepsin (Worthington Biochemical Corp.) as standard.

Results

Although cells washed and suspended in 0.05 *M* $MgCl_2$ solution (0.15 μ) failed on sonic treatment to give rise to an extract containing detectable isocitric dehydrogenase, an active enzyme resulted when 0.33 *M* K_2SO_4 (0.4 μ) was used. With extracts prepared with K_2SO_4 it was possible to show that the enzyme was TPN-linked, required Mg^{++}, and was non-specifically activated, like the aconitase of this organism, by a number of inorganic salts (2).

In an effort to determine why isocitric dehydrogenase could not be demonstrated in extracts when cells were washed with $MgCl_2$, cells from the same growth medium were divided into three parts and each part washed and suspended in either $MgCl_2$, $MgSO_4$, or K_2SO_4 solutions, all of the same ionic

strength. The extracts obtained on sonic treatment were then tested for isocitric dehydrogenase and aconitase activity under conditions previously established to be optimum for the activity of these enzymes (Table I). It is evident that

TABLE I

Isocitric dehydrogenase and aconitase activity of extracts prepared by washing and suspending cells in either $MgSO_4$, $MgCl_2$, or K_2SO_4 solutions before sonic treatment

Suspending solution			Specific activity*	
Solute	*M*	μ	Isocitric dehydrogenase	Aconitase
$MgCl_2$	0.05	0.15	8	377
$MgSO_4$	0.0375	0.15	1793	270
K_2SO_4	0.05	0.15	787	204

*Cuvette contents for isocitric dehydrogenase: *dl*-isocitrate (Tris salt), 20 μmoles; TPN, 1 μmole; $MgCl_2$, 50 μmoles; Tris buffer, pH 8.0, 320 μmoles. Total ionic strength in cuvette, ca.0.38. Cell extract, 0.05 to 0.1 mg protein. Total vol., 3.0 ml. Temp., 25° C.
Cuvette contents for aconitase: *dl*-isocitrate (Tris salt), 30 μmoles; Tris buffer, pH 8.0, 330 μmoles. Total ionic strength in cuvette ca. 0.42. Cell extract, 0.1 to 0.25 mg protein. Total vol., 3.0 ml. Temp., 25° C.

both $MgSO_4$ and K_2SO_4 solutions but not $MgCl_2$ gave rise to extracts containing appreciable isocitric dehydrogenase activity while all three preparations were active with respect to aconitase. This finding ruled out Mg^{++} and the ionic strength of the suspending medium as being factors responsible for the lack of isocitric dehydrogenase activity in extracts prepared from cells suspended in 0.05 *M* $MgCl_2$. Two other possibilities were considered, either that Cl^- was inhibitory or $SO_4^=$ stimulatory for the enzyme. The first could not be the case since only 5 μmoles of Cl^- were introduced into the reaction system with the extract while some 400 μmoles (300 from the Tris buffer and 100 from the $MgCl_2$) were present in the test system under conditions found to be optimum for demonstrating isocitric dehydrogenase activity. The second possibility was tested by washing and suspending cells in a potassium phosphate solution and comparing the isocitric dehydrogenase activity of the extract prepared with that of an extract from cells from the same growth medium washed and suspended in a K_2SO_4 solution. The results (Table II) show that an extract

TABLE II

Isocitric dehydrogenase activity of extracts from cells washed and suspended in various salt solutions

Suspending solution			
Solute	*M*	μ	Specific activity†
K_2SO_4	0.05	0.15	700
KH_2PO_4/K_2HPO_4	*	0.15	797
KCl	0.15	0.15	0
KBr	0.15	0.15	0
KI	0.15	0.15	0

*0.043 *M* KH_2PO_4, 0.026 *M* K_2HPO_4.
†Of isocitric dehydrogenase. For test system used, see Table I.

prepared from phosphate washed cells was quite as active as one from sulphate-suspended cells even when the extract was tested in a system containing no

added sulphate. Thus sulphate would appear to have no capacity to activate the system specifically. When other portions of cells from the same growth medium were washed and suspended in halide salt solutions, it is evident that the extracts obtained had no isocitric dehydrogenase activity. Neither of the three halide salts when tested at levels which would be introduced by the extract into the test system showed any capacity to inhibit the system when an extract containing an active enzyme was present.

Although the proper choice of a salt for use as the solute in solutions used to wash and suspend the cells for sonic treatment was essential to obtain isocitric dehydrogenase activity in the resulting cell-free extract, the ionic strength of the solution was found also to have a marked effect upon the activity of the enzyme released. It is clear from Fig. 1 that when a K_2SO_4 solution was

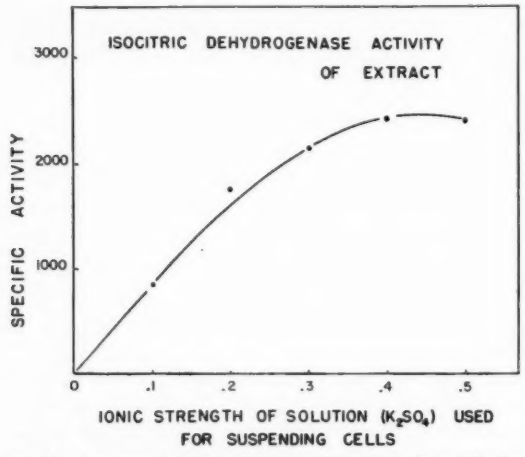

FIG. 1. Effect of ionic strength of medium used for suspending cells during sonic treatment on the activity of isocitric dehydrogenase in the resulting cell extract. Cuvette contents: *dl*-isocitrate (Tris salt), 20 μmoles; TPN, 1 μmole; $MgCl_2$, 50 μmoles; Tris buffer, pH 8. Total Tris concentration, 0.343 *M*. Ionic strength of cuvette contents, ca. 0.38. Cell extract, $\simeq$ 0.25 mg protein/ml. Total vol., 3.0 ml. Temp., 25°.

used to wash and suspend the cells, the specific activity of the extract prepared was nearly proportional to the ionic strength of the suspending solution. Maximum activity was obtained at an ionic strength of about 0.4. This effect of ionic strength during or prior to sonic treatment is independent of the ionic strength requirements for enzyme activation. The extracts prepared from cells suspended at each of the ionic strengths shown as points on the curve in Fig. 1 were in each case assayed for activity under conditions of the same ionic strength, which in this experiment meant adjusting the cuvette contents to 0.38 μ with Tris buffer.

Although KCl present as the sole solute in a solution used to suspend the cells did not give rise to an extract with significant isocitric dehydrogenase activity, it was found to be capable of contributing to the ionic strength

requirements for obtaining an extract with an appreciable specific activity when combined with a small amount of another salt able to give rise to an active enzyme (Table III). It is evident that a combination of KCl contributing 0.1 μ with K_2SO_4 contributing 0.05 μ gave rise to an extract with much

TABLE III

Capacity of a halide salt to contribute to the ionic strength requirements for activating isocitric dehydrogenase from a marine bacterium

Suspending solution		Protein conc.*	Specific activity†
Solute	μ		
K_2SO_4	0.05	13.8	540
	0.15	16.0	2260
	0.30	18.5	3020
KCl	0.10	16.5	22
	0.15	20.0	132
K_2SO_4+KCl	0.05+0.1	17.5	1225

*Of the extracts prepared, in mg/ml.
†Of isocitric dehydrogenase. For test system used, see Table I.

more activity than one prepared from cells suspended in 0.05 μ K_2SO_4 alone, though with less than in an extract from cells suspended in a K_2SO_4 solution of the same ionic strength as the combination.

Included in Table III are the protein concentrations of the extracts prepared from cells suspended in the different salt solutions. A recent study has shown that a certain minimum salt concentration is required to obtain disruption of cells by sonic treatment (10). It is evident, however, that the ionic strength effects obtained in this study were not related to the extent of cell disruption as measured by the protein concentrations in the various extracts.

The results obtained so far suggested that there were two requirements for obtaining an active isocitric dehydrogenase. One was that a halide not be the sole anion in the medium used to suspend the cells, the other that the medium be of appropriate ionic strength. Since the halide effect appeared not to be a positive inhibitory one it seemed possible that it was due to an inability of halide to protect the enzyme during preparation either from physical stresses imposed during sonic treatment, from one or more components of the extract which might be capable of destroying it, or from spontaneous denaturation.

To determine if the halide effect was specific for extracts prepared by sonic disruption of the cells, extracts were also prepared by two other methods, alumina grinding and lysis by versene and lysozyme. Cells from a medium were divided into two portions, one of which was washed and suspended in a K_2SO_4, the other in a KCl solution. Half of each suspension was then used to prepare a cell-free extract by alumina grinding and half was treated sonically to provide a control. In a separate experiment, extracts prepared by cell lysis were also compared with a control obtained by sonic treatment.

In both cases (Table IV) extracts from cells washed and suspended in the halide solution had little or no isocitric dehydrogenase activity while those from cells suspended in a K_2SO_4 solution were very active. Aconitase activity,

measured in the extracts obtained by cell lysis, was again essentially independent of the solutions used to suspend the cells.

TABLE IV

Comparison of enzyme activity in extracts prepared by alumina grinding, lysis by versene and lysozyme, and sonic treatment from cells suspended in solutions of either KCl or K_2SO_4

Method of preparation	Suspending solution	Specific activity†	
		Isocitric dehydrogenase	Aconitase
Alumina grinding	K_2SO_4	2400	—
	KCl	98	—
Sonic	K_2SO_4	1935	—
	KCl	163	—
Lysis*	K_2SO_4	1876	296
	KCl	0	418
Sonic	K_2SO_4	1877	236
	KCl	97	286

*Lysis by versene and lysozyme.
†For test systems used, see Table I.

Since results obtained with extracts prepared by cell lysis were similar to those found when extracts were prepared by less gentle procedures, it seemed unlikely that physical stresses during preparation of the extract could be responsible for the halide effect. A possible clue to the nature of the phenomenon resulted from the observation that upon occasion an extract prepared from cells washed and suspended in a halide solution did have isocitric dehydrogenase activity. Under such circumstances the enzyme was very unstable and lost most of its activity after storage for 24 hours at 4° C. When the rate of loss of activity of such a preparation was compared with that of a 1:10 dilution of the same preparation, it was found that there was very little difference (Table V). Had the loss of activity been due to the presence in the extract of an agent such as a proteolytic enzyme or heavy metal ion capable of catalytically inactivating the isocitric dehydrogenase and against which sulphate ion pro-

TABLE V

Effect of dilution on rate of loss of isocitric dehydrogenase from extracts prepared from cells suspended in either $MgCl_2$ or $MgSO_4$ solutions

Suspending medium*	Dilution of extract†	Storage time (hr)‡		
		0	24	72
		Activity remaining (%)		
$MgCl_2$	None	100	27	1
	1:10	100	13	1
$MgSO_4$	None	100	85	73
	1:10	100	—	83

*$MgCl_2$ solution 0.05 *M* (0.15 μ), $MgSO_4$ 0.0375 *M* (0.15 μ). Extracts prepared by sonic treatment.
†Dilutions made with solutions used to suspend the cells.
‡Extracts stored at 4° C.

vided protection, dilution of the extract should have reduced the rate of loss of activity appreciably. As it was, however, the rate of loss of activity was essentially independent of the concentration of the enzyme and would thus appear to be following the kinetics of a first order reaction. One must conclude then that the enzyme is spontaneously denaturing and that this process proceeds very much more readily in a halide solution.

Another inorganic factor affecting the stability of isocitric dehydrogenase came to light in the course of this study. It was found that the presence of Mg^{++} in the solution considerably improved the stability of the enzyme on storage under conditions considered otherwise optimum for maintaining the activity of the enzyme. This is shown in Table VI where the addition of 0.05 *M*

TABLE VI

Capacity of Mg^{++} to stabilize isocitric dehydrogenase in a cell-free extract of a marine bacterium

Suspending medium*	Storage time (days)						
	0	1	2	5	6	13	20
	Activity remaining (%)						
K_2SO_4	100	72	48	28	13	0	0
$K_2SO_4+MgSO_4$	100	92	72	60	56	16	10
$K_2SO_4+MnSO_4$	100	91	64	27	19	0	0

*Cells from the same medium were washed and suspended in 0.133 *M* K_2SO_4 (0.4 μ) for sonic treatment. The extract was divided into three portions. Two of these were made 0.05 *M* with respect to either $MgSO_4$ or $MnSO_4$. To the third, sufficient additional K_2SO_4 was added to compensate for the increased ionic strength resulting from the additions to the other two portions. Extracts were stored at 4° C and aliquots diluted appropriately with 0.1 *M* K_2SO_4 for testing. Test system used, see Table I.

Mg^{++} to a cell-free extract prepared from cells washed and suspended in a K_2SO_4 solution, considerably reduced the rate of loss of isocitric dehydrogenase activity in the preparation. Mn^{++}, which can replace Mg^{++} in activating the enzyme, showed no significant capacity to replace Mg^{++} in stabilizing it.

Discussion

There would appear to be three separate effects of inorganic salts on the stability of the isocitric dehydrogenase in this organism. First, there is the non-specific requirement for a medium of appropriate ionic strength to maintain the activity of the enzyme. Superimposed on this is the more specific need for sulphate or phosphate ions as opposed to halides to prevent loss of activity. Finally, when both of the above requirements are met, the presence of Mg^{++} causes additional stabilization of the enzyme.

The data indicate that loss of activity of isocitric dehydrogenase when a halide is the sole anion in the medium is most probably due to protein denaturation. It is likely that the other ion effects protect against this same phenomenon.

Enzymatic activity in a protein appears to depend in some way on the manner in which the polypeptide chain and its attached amino acid residues are arranged within and on the surface of the molecule, the whole structural pattern being stabilized by a great number of weak secondary intramolecular bonds

(11). The fact that three separate effects of ions on the stability of the isocitric dehydrogenase reported here have been distinguished suggests that there are at least three separate ways in which the intramolecular bond forces in this protein can be modified to bring about protein denaturation.

Specific effects of anions on the stability of proteins have been reported. Denaturation of ovalbumin in urea solutions was inhibited strongly by such multiply charged anions as sulphate and phosphate and to a much lesser extent by halides (12). Denaturation of ovalbumin by heat, on the other hand, was strongly inhibited by *both* Na_2SO_4 and NaCl (13). Differences in the capacity of anions to activate an enzyme have also been reported (14).

It was at first considered possible that the ability of Mg^{++} to stabilize the isocitric dehydrogenase was related to its capacity to activate the enzyme, possibly by becoming bound at and thus protecting a labile group at the active site. This was rendered less likely by the observation that Mn^{++}, which also activates the enzyme, was unable to stabilize it. Furthermore, it has not been established whether the ability of Mg^{++} and Mn^{++} to activate isocitric dehydrogenase in the crude extract is in reality due to the requirement of an oxalosuccinic carboxylase for these ions as is the case with the pig heart enzyme (7). It may be of significance in connection with the Mg^{++} effect that $CaCl_2$ was found to be much more effective than chlorides of monovalent cations in preventing denaturation of the lactic dehydrogenase from a halophilic bacterium (15). Unfortunately, Ca^{++} could not be tested in the system reported here because of the attendant requirement for sulphate or phosphate for stability. That one cannot generalize regarding the capacity of alkaline-earth cations to prevent denaturation, however, is evident from the fact that Ca^{++}, Mg^{++}, and Ba^{++} salts have been observed to accelerate the denaturation of ovalbumin in urea solutions (11).

In one recent study, isocitric dehydrogenase activity could not be demonstrated in extracts of *Acetobacter peroxidans* (16). Based on this observation and failure to demonstrate the oxidation of citrate and α-ketoglutaric acid, it was concluded, with reservations, that terminal acetate oxidation in this organism takes place by a dicarboxylic rather than by a tricarboxylic acid cycle. In another study with the same organism, evidence for isocitric dehydrogenase activity was obtained (17). In the investigation where isocitric dehydrogenase could not be detected, cells of the organism had been washed and suspended in water prior to sonic treatment, while where it had been found, the cells had been washed and suspended in phosphate buffer. In view of the present findings, it seems not unlikely that this difference in procedure could have been responsible for the discrepancy in the results obtained.

It would appear that there is no way of predicting the capacity of electrolytes to affect the activity or stability of an enzyme. Awareness of their possible importance in permitting the detection of an enzyme is desirable, however, when conclusions regarding the pathway of metabolism in an organism is to be based on failure to detect a key enzyme.

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THYROID ENLARGEMENT PRODUCED BY PROLONGED ADMINISTRATION OF IODINE IN THE MOUSE[1]

H. ISLER,[2] N. J. NADLER, T. ARBOIT, N. JOHNSTON, W. PETER, AND E. TUCKER

Abstract

Mice were fed diets containing a low level of sodium chloride and 0.2, 1.0, and 5.0 μg iodine per gram respectively for 72 and 142 days. At 142 days, the thyroids weighed 1.6, 3.0, and 4.2 mg per lobe respectively. To determine which thyroid tissue components were responsible for the increase in gland weight, quantitative measurements were performed on histological sections of the thyroid. At the lowest and highest iodine levels, the colloid weights were 1.0 and 3.0 mg per lobe and the epithelium weights were 0.39 and 0.88 mg per lobe respectively. Thus, the change in colloid accounted for 77% and the change in epithelium for 19% of the increase in thyroid weight. Follicular diameter increased by 40%. The goiter induced by iodine proved to be due essentially to an enlargement of follicle size and not to an increase in the number of follicles. Kidney inflammation was observed in animals ingesting large amounts of iodine.

It has long been known that a low iodine diet produces goiter in animals but it was only recently discovered that raising the iodine content of the diet can also result in an increase in thyroid weight when the intake of sodium chloride is low (1). Thus, in mice, levels of 0.12 and 0.32 μg iodine per gram diet maintained the weight of the thyroid at a minimum, while a level of 1 μg per g produced an increase in thyroid weight from 1.0 to 2.5 mg per thyroid lobe (1).

In animals fed a low iodine diet, the histology of the goiter is well recognized: the amount of colloid diminishes and the cells hypertrophy and increase in number. However, in animals fed a high iodine diet, the corresponding histological changes associated with the increase in thyroid weight have not heretofore been described.

In the work now to be reported, the observation that high levels of iodine do produce an increase in thyroid weight was confirmed, and the thyroid tissue components responsible for this increase were investigated.

Material and Method

Male C_3H mice, 3½ months old at the start of the experiment, were fed a diet containing 0.01% sodium chloride, 1% calcium carbonate, 10% brewer's yeast, and 88.99% corn meal. The basic iodine content of this diet, which was 0.06 μg/g, was raised by addition of sodium iodide to, respectively, 0.2, 1.0, and 5.0 μg per g. Three groups of 17 mice each were fed the three diets respectively. Food and distilled water were given ad libitum. In each group, six animals were sacrificed at 72 days and the rest at 142 days.

Two and a half hours before sacrifice, each mouse was injected subcutaneously with 10 μc I^{131}. The urine was collected during the 2½ hour period. At the time of sacrifice, the left thyroid lobe was dissected free from connective tissue

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and weighed on an analytical balance to the nearest 10th of a milligram. The radioiodine content of the left thyroid lobe and urine were estimated according to techniques described previously (2). The right thyroid lobe still attached to the trachea was fixed in Bouin, sectioned, and stained with periodic acid – Schiff and haematoxylin.

The proportion of the thyroid gland occupied by colloid was estimated in one histological section of each thyroid by a modification of the so-called "line" method of Uotila (3, 4). The relative amounts of colloid, epithelium, and connective tissue were determined according to a modification of the "random hit" method of Chalkley (5). An ocular grid, consisting of a set of perpendicular lines, was used, and the intersection of the lines determined the hits. In each animal an entire thyroid section was scanned, and 300 to 500 hits were recorded.

The diameters of the follicle sections were measured from basement membrane to basement membrane in two directions at right angles. From these measurements, the true follicle size distribution was calculated by the statistical method of Nadler, Leblond, and Bogoroch (6).

Results

Body weight was not influenced by dietary iodine (Table I). Radioiodine uptake by the thyroid was less in the mice receiving the highest level of iodine, while no change was observed in the urinary secretion of radioiodine. The thyroid weight was significantly increased from 1.8 to 2.8 mg per lobe at 72 days and from 1.6 to 4.2 mg at 142 days, when iodine level in the diet was raised from 0.2 to 5.0 μg per g (Table I).

Although the thyroids showed large individual variations, histometric measurement by the "line" method revealed that the proportion of colloid was significantly increased by iodine. Assuming the same specific gravity for all tissue components, it was calculated that the increase in thyroid weight was due mainly (about 75%) to an increase in colloid mass, but also (about 25%) to the other tissue components (Fig. 1). To obtain more information on the effects of dietary iodine on the other components, the proportion of epithelium, connective tissue as well as colloid, was estimated by the "random hit" method. As far as colloid is concerned this technique yielded essentially the same results as the line method; that is, it revealed an increase in the proportion and in the absolute mass of colloid, with greater iodine intake (Table I). In addition, it showed that in mice fed for 142 days on the 5.0 μg iodine per gram diet, the absolute mass of the epithelium was about twice that in animals fed the diet containing 0.2 μg iodine per gram. The size of the follicles was increased by iodine as indicated by their average diameter (Table I) and by the shift observed in their size distribution (Fig. 2). Iodine treatment did not change significantly the total mass of connective tissue (Table I).

Thus, a high level of dietary iodine produced a goiter, due mainly to an increase in colloid, but also partly to an increase in the epithelium mass.

It was noted, incidentally, that after 72 days the mice ingesting the highest amount of iodine possessed enlarged and pale kidneys with a granular surface. In each of the three groups the kidneys of two animals were weighed. They

TABLE I

Effect of iodine intake on the thyroid gland of mice.
Duration of experiment 72 and 142 days

	μg iodine per g diet	72 days		142 days	
		Number of mice	Mean ± S.E.	Number of mice	Mean ± S.E.
Body weight (g)	0.2	6	21.4±1.22	6	25.9±0.8
	1.0	5	23.3±0.70	5	24.9±0.5
	5.0	6	22.6±0.89	5	25.7±0.5
% of injected I^{131} per thyroid lobe	0.2	6	1.9±0.7	6	4.2±0.6
	1.0	5	1.8±0.5	5	3.5±1.1
	5.0	6	0.7±0.2	5	*1.1±0.2**
% of injected I^{131} in urine	0.2	6	4.5±1.3	6	7.1±1.6
	1.0	5	4.3±0.6	5	7.9±1.0
	5.0	6	5.5±0.8	5	7.0±1.1
Thyroid lobe weight (mg)	0.2	6	1.8±0.1	6	1.6±0.3
	1.0	5	1.8±0.3	5	3.0±0.6
	5.0	6	*2.8±0.3**	5	*4.2±0.6**
Colloid weight per thyroid lobe (mg)	0.2	5	1.0±0.1	5	1.0±0.3
	5.0	6	*1.8±0.2**	5	*3.0±0.5**
Epithelium weight per thyroid lobe (mg)	0.2	5	0.51±0.08	5	0.39±0.09
	5.0	6	0.62±0.07	5	*0.88±0.12*
Connective tissue weight per thyroid lobe (mg)	0.2	5	0.26±0.03	5	0.20±0.05
	5.0	6	0.32±0.06	5	0.35±0.06
Diameter of follicular section (μ)	0.2	—	—	5	71.0±3.0
	5.0	—	—	5	*101.7±3.6**
True diameter of follicle (μ)	0.2	—	—	5	89.9±1.4
	5.0	—	—	5	*125.8±1.9*

*Significantly different from the corresponding figures obtained with the lowest iodine level.

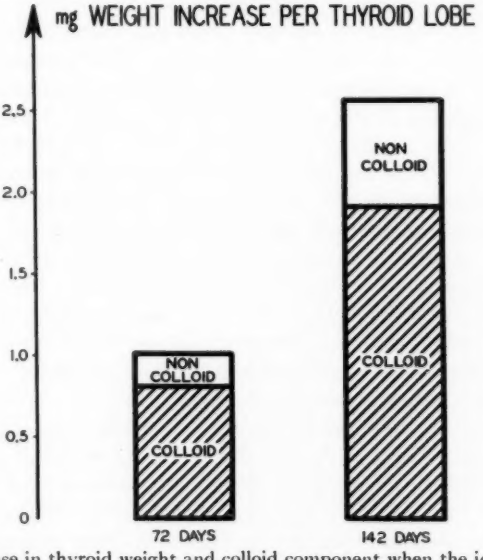

FIG. 1. Increase in thyroid weight and colloid component when the iodine level in the diet was raised from 0.2 to 5.0 μg per gram, after 72 and 142 days' treatment. About 75% of the increase in thyroid weight is due to colloid.

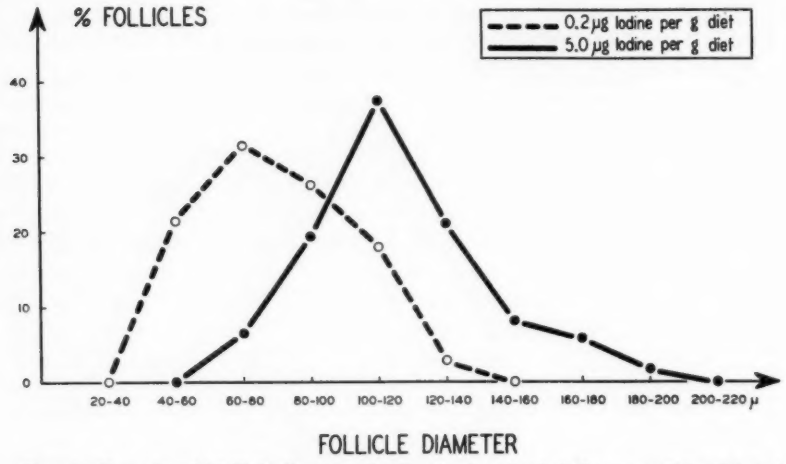

FIG. 2. True thyroid follicle diameter distribution in mice fed diets containing 0.2 and 5.0 μg iodine per gram respectively, for 142 days. The follicles are larger at the higher iodine level.

averaged 150, 189, and 247 g respectively with increasing iodine level. Histologically the kidneys showed hemorrhagic areas, and their tubules contained casts. Leukocytes were not conspicuous. At 142 days, these pathological features were also present.

Discussion

The present experiment shows that prolonged ingestion of relatively large, although not pharmacological doses, of iodine may definitely lead to thyroid enlargement. It is to be noted that this effect has been obtained when the animals had been fed a diet containing a very low level of sodium chloride. In fact it is known that, in the presence of higher levels of this salt, the goiter is not as large (1), due to the fact that salt does considerably raise the renal clearance of the iodide ion (2), and therefore reduces the level of circulating iodide. The goitrogenic effect of iodine has been suspected in man (7, 9), while in animals only an increase in colloid and flattening of the epithelium has been reported (9). In the present work, the colloid mass augmented and was actually responsible for much of the increase in thyroid weight. However, the epithelium mass also increased as much as twofold. Theoretically, these changes can be accounted for either by an increase in the number of follicles, or by an increase in the size of the follicles present, or by both.

It was determined that iodine did actually induce an enlargement of the size of the follicle. The true follicle diameter, computed from the diameters measured in histological sections, showed an increase from 89.9 μ at 0.2 μg iodine per g diet, to 125.8 μ at 5.0 μg, after 142 days on the diet. Assuming spherically shaped follicles, this 40% increase in *diameter* corresponds to a 175% increase in *volume*. Since the total thyroid weight showed a very similar increase (163%), the goiter can be entirely accounted for by an increase in

follicular size, and it is not necessary to postulate an increase in the number of follicles.

Similarly, a 40% increase in the diameter of a sphere would result in a 96% gain in surface area, occupied, in the case of the thyroid follicle, by the epithelium. The actual gain in epithelium mass was $126 \pm 44\%$ (standard error calculated according to the method of Fenner (10)), which is not significantly different from the expected 96% figure. Considering that the height of the epithelium was changed little, this is in agreement with the conclusion that iodine treatment produces essentially a growth of the existing follicles, without inducing the formation of new ones.

Acknowledgments

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METABOLIC EFFECTS OF UREA ADMINISTRATION ON ACUTE HYPOTHERMIA IN RATS[1,2]

JOHN R. BEATON

Abstract

Acute hypothermia (15° C rectal temperature) has been induced in fasted rats with and without prior intraperitoneal administration of urea. In the hypothermic animal, not given urea, blood glucose and pH were significantly decreased; blood lactic acid, inorganic phosphorus, and packed cell volume were significantly increased; no alterations occurred in blood levels of pyruvic acid, total acid-soluble phosphorus, urea, nor amino nitrogen. Prior administration of urea prevented significant alterations of blood glucose and lactic acid in the cooled animal. In the non-cooled animal, urea treatment resulted in elevated blood glucose levels. These effects are discussed in relation to the beneficial action of urea in permitting successful resuscitation and survival in a normal condition of rats cooled to rectal temperatures of 0–3° C.

Introduction

In a recent paper from this laboratory (1), it was reported that intraperitoneal administration of either acetazoleamide or urea in 0.85% aqueous sodium chloride prior to cooling was successful in permitting resuscitation and survival of rats in an apparently normal condition after cooling under ice to a rectal temperature of 0–3° C. Although we successfully resuscitated some of the animals injected with 0.85% aqueous sodium chloride alone, marked paralysis was noted, particularly in the hind quarters. Similarly, intraperitoneal administration of the organic buffer 2-amino-2(hydroxymethyl)1,3-propane diol (THAM) in 0.85% aqueous sodium chloride, although permitting resuscitation of some animals, resulted in a marked paralysis of the hind quarters. In the absence of any pretreatment, successful resuscitation and survival of cooled animals did not occur.

As part of long-term studies in this laboratory on effects of acute hypothermia, biochemical changes occurring in rats as a result of hypothermia per se and the extent to which these are affected by administration of urea prior to cooling have been investigated and reported herein.

Materials and Methods

In all cases, adult female albino rats of the Wistar strain, maintained on fox chow and drinking water ad libitum, were used. Food was withdrawn 18 hours before experiment to eliminate the variable effects of food ingestion on blood metabolite levels. Control animals were given 3.0 ml of 0.85% aqueous sodium chloride by intraperitoneal injection; urea-treated animals received 120 mg urea/100 g body weight contained in 3.0 ml of 0.85% aqueous sodium chloride by intraperitoneal injection. In all cases, a period of 8 minutes was allowed to elapse between injection and initiation of cooling to permit absorption of urea yielding a significantly elevated blood urea level as determined in preliminary

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experiments. Cooling was carried out by placing unanesthetized animals in individual, cylindrical screen cages under ice until a rectal temperature of approximately 15° C was attained, this requiring a period of about 25 minutes. At this temperature, respiration had almost ceased and cardiac activity was very markedly reduced. Our experience has been that further cooling to a rectal temperature of 8–10° C or less results in cessation of cardiac activity and difficulty is encountered in withdrawing an adequate blood sample for analysis of the metabolites under study. Blood samples were withdrawn by syringe from the exposed heart (right ventricle) following intraperitoneal injection of sodium pentobarbital in 0.85% aqueous sodium chloride at a level of 16 mg/100 body weight to effect rapid anesthesia since slowly acquired anesthesia might cause alterations in blood metabolite levels. Care was taken to avoid unnecessarily increasing activity of the rats during these studies since this alone would alter levels of the blood metabolites under study. The blood was heparinized and a trace of sodium fluoride was added to each blood sample to stop glycolysis. Protein-free filtrates were prepared immediately and analyzed by the following methods: glucose, Folin and Wu (2); lactic acid, Barker and Summerson (3); pyruvic acid, Friedemann and Haugen (4); inorganic phosphorus and total acid-soluble phosphorus, Fiske and Subbarow (5); urea, Archibald (6); amino nitrogen, Frame, Russell, and Wilhelmi (7); packed cell volume, Wintrobe (8). Determinations of pH were carried out immediately on blood samples employing a Metrohm pH meter with Sanz-Metrohm capillary electrode adjusted to the rectal temperature of the rat by circulating temperature-controlled water through a surrounding water jacket. In agreement with Gambino (9) it was determined that heparin in the amount used had no effect upon blood pH. Further we have noted no significant effect on pH of sodium fluoride in the trace amounts used.

Blood Packed Cell Volumes in Urea-treated Cooled and Non-cooled Rats

Four groups of nine rats each were used to compare the effect of intraperitoneal administration of urea with that of saline on packed cell volumes in cooled and non-cooled animals. In the non-cooled rats, blood samples were taken 33 minutes after injection of urea or of 0.85% aqueous sodium chloride so that, on a time basis, these animals were comparable to the ones cooled for 25 minutes, 8 minutes after injection.

Blood Urea Curves in Cooled and Non-cooled Rats

Blood urea levels were determined at intervals of time following urea administration to cooled and non-cooled rats weighing 200–250 g. Each group consisted of eight animals. In this experiment, one group of cooled animals was resuscitated by rapid rewarming in a 40° C water bath accompanied by forced ventilation with 95% oxygen – 5% carbon dioxide as previously described (1).

Effects of Hypothermia and/or Urea on Fasting Levels of Blood Metabolites

Three groups of nine rats each were used: a control, non-cooled group; a control, cooled group; a urea-treated, cooled group. Measurements of pH and of several blood metabolites were made.

The Effect of Urea Administration on Blood Glucose Levels in Rats

The effect of urea administration on blood glucose levels in non-cooled rats was investigated. Seven groups of eight male, fasted rats weighing 200–250 g each were randomized and treated as follows. Three groups received aqueous sodium chloride and three groups received urea in aqueous sodium chloride. Two groups were sacrificed at each of 30, 60, and 120 minutes after injection. One group was sacrificed as an initial control.

Results

Blood packed cell volumes, expressed as mean ± standard deviation, following urea administration in hypothermic and non-cooled rats are shown in Table I. From the results it is evident that, under these experimental condi-

TABLE I

Effect of urea injection on blood packed cell volumes in non-cooled and cooled rats (Results expressed as mean ± standard deviation for nine rats)

Group	Body weight, grams	Rectal temperature, °C	Blood packed cell volume, %
Control	237 ± 21	—	48 ± 3
Urea-treated	243 ± 22	—	47 ± 2
Control, cooled	268 ± 9	13 ± 2	55 ± 4
Urea-treated, cooled	238 ± 27	14 ± 2	58 ± 5

tions, urea administration had no significant effect upon blood packed cell volumes in non-cooled rats nor upon the significant elevation in packed cell volumes consequent upon induced hypothermia when compared with saline-injected controls. Significant elevations in blood packed cell volumes associated with hypothermia have been commonly observed by workers in the field. From these results, it can be concluded that differences in blood metabolite levels following urea administration are not due to alterations in hemoconcentration.

Blood urea levels following urea administration to non-cooled and cooled rats are shown in Fig. 1. It is evident that a marked elevation in blood urea levels had occurred 8 minutes after injection of urea, i.e., at the time that cooling was begun. Blood urea levels thereafter followed essentially the same pattern in cooled and non-cooled rats though at a significantly higher level in the cooled rats. This significantly higher level in cooled animals ($P < 0.001$) might be attributable to cessation of renal excretion at a rectal temperature of approximately 18° C (10). The blood urea level of urea-injected rats which had been cooled and then resuscitated was still significantly higher than that of saline-injected rats at the same time interval ($P < 0.01$).

As shown by the data in Table II, hypothermia per se resulted in significantly lower blood glucose levels accompanied by significantly higher levels of blood lactic acid and inorganic phosphorus (whether expressed as mg% or as % of acid-soluble phosphorus). No significant alterations were noted in blood levels of pyruvic acid, acid-soluble phosphorus, urea, nor amino nitrogen as a consequence of hypothermia. As commonly observed by others (11, 12) hypothermia

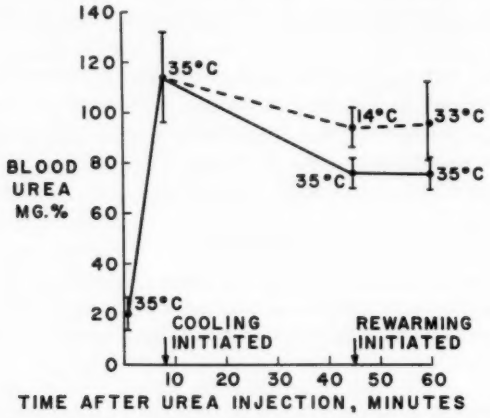

FIG. 1. Blood urea levels in cooled (●——●) and non-cooled (●- - -●) rats (200–250 g) following intraperitoneal injection of urea (120 mg/100 g). Each point represents the mean ± standard deviation for eight rats. Mean rectal temperature of the group is indicated at each point.

TABLE II

Effect of intraperitoneal injection of urea on blood pH and metabolite levels in hypothermic rats (Results expressed as mean ± standard error of the mean for nine rats/group)

Group	Control, non-cooled (1)	Control, cooled (2)	Urea-treated, cooled (3)	Level of significance, P*		
				(1) vs. (2)	(1) vs. (3)	(2) vs. (3)
Body weight, g	209±7.0	224±5.3	238±5.3	—	—	—
Rectal temp., °C	34±0.2	16±0.2	15±0.7	<0.001	<0.001	—
Blood pH, units	7.34±0.018	7.06±0.021	7.05±0.130	<0.01	<0.01	—
Blood glucose, mg%	100±6.7	85±3.0	106±8.7	<0.025	—	<0.05
Blood lactic acid, mg%	67±4.3	94±4.3	80±5.0	<0.001	—	<0.05
Blood pyruvic acid, mg%	1.6±0.20	1.6±0.07	1.9±0.07	—	—	—
Blood inorganic phosphorus, mg%	5.3±0.10	11.9±0.90	11.5±0.63	<0.001	<0.001	—
Blood inorganic phosphorus, % of acid-soluble phosphorus	21±2.3	40±3.7	41±4.0	<0.01	<0.001	—
Blood acid-soluble phosphorus, mg%	25±2.3	30±3.0	28±2.3	—	—	—
Blood urea, mg%	20±2.0	19±1.7	74±5.0	—	<0.001	<0.001
Blood amino nitrogen, mg%	9.5±0.30	9.8±0.8	8.1±0.23	—	<0.01	—

* Probabilities calculated by application of "Student's" t test.

resulted in a lowering of blood pH. In the hypothermic rats pretreated with urea, blood levels of glucose and lactic acid were not significantly different from those of the non-cooled rats. Significantly lower blood amino nitrogen levels during hypothermia resulted from urea administration. Urea was without effect on the cold-induced increase in blood inorganic phosphorus nor did it prevent the decrease in blood pH resulting from cooling.

Blood glucose levels following urea administration to fasted, non-cooled rats are shown in Fig. 2, each point representing the mean ± standard deviation for eight animals. The increase in blood glucose following urea injection was significant at 30, 60, and 120 minutes compared to the initial level (P <0.001) and the levels were significantly higher than those of saline-injected controls at 30, 60, and 120 minutes (P <0.01, <0.001, and <0.01 respectively).

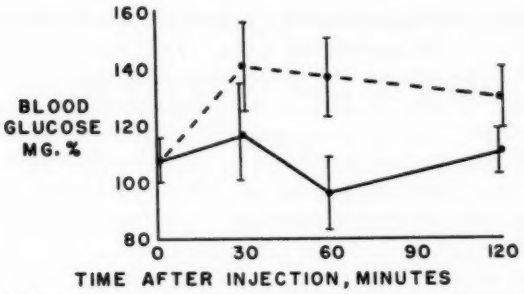

FIG. 2. Blood glucose levels following intraperitoneal injection of urea (●- - -●) and of 0.85% aqueous sodium chloride (●———●). Each point represents the mean ± standard deviation for eight rats.

Discussion

It should be emphasized that these studies were carried out under specific conditions of acute hypothermia using fasted animals. Unlike the studies of others, neither anesthesia nor artificial ventilation were used during the cooling phase in these investigations although sodium pentobarbital was administered just prior to withdrawal of cardiac blood. Blood glucose, lactic acid, and pyruvic acid levels in hypothermia can be expected to vary with experimental conditions of hypothermia induction. As an example, Fisher, Fedor, and Lee (13), studying hypothermia in dogs, noted hyperglycemia at the outset of cooling, changing to hypoglycemia as cooling was prolonged. Fuhrman and Crismon (14) observed that, in shivering rats provided with ample carbohydrate prior to cooling, blood glucose levels rose, whereas in starved animals or slowly cooled, fed animals, blood glucose levels were maintained or decreased. In the absence of shivering, the blood glucose level was maintained or decreased. Henneman *et al.* (15) have noted that during hypothermia in man, without shivering and with induced hyperventilation, there is a decrease in blood lactic acid, pyruvic acid, and inorganic phosphorus with a moderate rise in blood glucose concentration, suggesting a failure of glucose to enter the metabolic pool under their experimental conditions.

In the present experiment, the lowered blood glucose levels accompanied by elevated blood lactic acid and inorganic phosphorus levels in hypothermic rats indicate that glucose, and possibly glycogen, are being metabolized. It appears that formation of lactic acid is increased, its oxidative catabolism, presumably through pyruvic acid, is decreased, or both are altered in rats whose body temperature has been lowered to 15° C. It is suggested that in hypothermic animals, the available oxygen is insufficient for the animals' reduced metabolic activity. Kaplan (16) in an excellent summary of the knowledge relating phosphorus to carbohydrate metabolism has stated that an accumulation of inorganic phosphorus accelerates metabolism of carbohydrate and is unfavorable to glycogen synthesis. In this respect, the finding of increased levels of blood inorganic phosphorus in hypothermic rats at a body temperature of 15° C is of interest. Swan *et al.* (17) have reported that, under conditions differing from those reported here, serum phosphorus decreases slightly on

cooling. Fleming (18) observed no alteration in serum phosphorus in dogs cooled to a rectal temperature of 20° C.

It is difficult to explain why the blood levels of glucose and lactic acid were not significantly different from normal in hypothermic rats pretreated with urea although high levels of blood inorganic phosphorus were found in these animals. These observations might conceivably indicate a direct effect of urea on carbohydrate metabolism or an indirect effect such as through alterations in cell permeability, in diffusion of metabolites, in electrolyte distribution, or in fluid distribution.

The increase in blood glucose in non-cooled animals after intraperitoneal injection of urea, although unexplained, is of interest in view of the significantly higher blood glucose levels of urea-treated cooled rats on comparison with cooled rats not given urea (Table II). In other studies in this laboratory we have noted an even greater blood glucose response to urea injection in non-fasted rats.

The reported results provide data on biochemical alterations occurring during acute hypothermia in rats under these specific conditions and as affected by prior urea administration, although they do not explain the apparently beneficial effect of urea pretreatment in hypothermic rats (1). It is possible that prevention of hypoglycaemia in cooled rats by prior treatment with urea may predispose to successful resuscitation. Further studies are in progress using larger laboratory animals to permit serial measurements of blood metabolites in the same animal. Current studies will extend this work also to the assessment of other metabolic processes in the hypothermic animal, particularly electrolyte alterations.

Acknowledgment

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DEGRADATION BY HYDRAZINE OF BOVINE PLASMA ALBUMIN AND LYSOZYME[1]

MADELEINE CHAMPAGNE[2] AND DAVID B. SMITH

Abstract

The effect of hydrazine and hydrazine hydrate on bovine plasma albumin and lysozyme at 5° and 20° C has been studied by viscosity, sedimentation, and molecular weight measurements. The appearance of new N-terminal amino acids and peptides has been demonstrated. The effect of these reagents is an initial unfolding of the molecule followed by slow, non-random fission to smaller particles.

Introduction

Rees and Singer (1) have found hydrazine to be a good solvent for many proteins but noted viscosity changes in hydrazine solutions of γ-globulin on standing. These investigators left open the question of whether these changes were due to alterations in the shape of the molecule or to breaking of covalent bonds or both. In the present study the effects of hydrazine and of hydrazine hydrate on bovine plasma albumin, both native and oxidized with performic acid (2), are described. These effects have been followed by measurements of viscosity, sedimentation velocity, and molecular weight and by identification of the N-terminal amino acids. Some experiments on lysozyme treated with hydrazine are also reported.

Material and Methods

Materials

Crystalline bovine plasma albumin (BPA), Lot No. S68108, and lysozyme, Lot No. 003L1, were obtained from the Armour Laboratories.

Oxidized BPA was made both by the method of Hirs (3) at −7° C and by the method of Reichmann and Colvin (2) at room temperature.

Hydrazine, 95+%, and hydrazine hydrate, 85% solution, were obtained from Eastman Organic Chemicals and Fisher Scientific Company respectively.

Dinitrophenyl- (DNP-) amino acids were obtained from Mann Research Laboratories or were prepared by established methods (4).

Solutions of protein in hydrazine or hydrazine hydrate were prepared and stored either at 5° C or 20° C.

Physical Methods

Viscosity measurements were made at 20° C in an Ostwald-Fenske viscometer (ASTM No. 50) with flow times for hydrazine and hydrazine hydrate of 230 and 460 seconds respectively. Solutions were filtered before being placed in the viscometer and remained in the viscometer, protected from atmospheric moisture, during an experiment.

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Sedimentation coefficients ($s_{20,w}$) were measured in a Spinco Model E ultracentrifuge at 20° C.

Molecular weights (M) were measured in the ultracentrifuge by Archibald's method, but before disappearance of the plateau region (5). Rotor speeds of 25,980 and 42,040 r.p.m. were used. Base lines were established by separate runs using solvent only and were found to be indistinguishable from extrapolation of the plateau region ($dn/dr = 0$). Second moments of the sections of the schlieren patterns between meniscus and plateau region about the axis of rotation, $\int_{r_m}^{r_p} r^2(dn/dr)dr$, were evaluated using a mechanical integrator (6). Here r is the distance from the axis of rotation, subscripts m and p indicate the meniscus and plateau values respectively, n is the refractive index difference between solvent and solution at any given level in the ultracentrifuge cell.

The slope of the line obtained by plotting $K/r_m\omega^2.(dn/dr_m)$ as ordinate against $K\int_{r_m}^{r_p} r^2\,(dn/dr)dr/{r_m}^2$ as abscissa is equal to $\{-M\,(1-\bar{v}\,\rho)\}/RT$ (7). K is the collected constants of the apparatus, ω is the angular velocity, $\bar{v}$ partial specific volume, ρ density of solvent, R gas constant, and T absolute temperature. The value 0.734 was used for $\bar{v}$ (8). The abscissal intercept of this line is Kn_0. The refractive index difference between the original solution and the solvent, n_0, was measured on a Phoenix-Brice-Speiser differential refractometer.

High voltage paper electrophoresis was carried out with an apparatus resembling that of Ryle *et al.* (9) using the pyridine – acetic acid buffer, pH 6.3, of Michl (10).

Chemical Methods

N-terminal amino acids were identified as their DNP-derivatives. After removal of hydrazine from the protein *in vacuo* over H_2SO_4, residual hydrazine and hydrazide groups were reacted with 20% butyraldehyde in water for 30 minutes. The preparation was dried and treated in bicarbonate buffer with 1-fluoro-2,4-dinitrobenzene (FDNB) (4). The ether extract of the acidified reaction mixture was designated extract I and of the acid hydrolyzate of the reaction product, extract II. The aqueous phase after the last extraction was dried and the residue was extracted with acidified n-butanol.

DNP-amino acids, if any, in the above extracts were separated by two dimensional paper chromatography using successively the tertiary amyl alcohol procedure of Blackburn and Lowther (11) and the 1.5 M pH 6 phosphate buffer of Levy (12). The yields of separated DNP-amino acids were estimated spectrophotometrically (4).

To see if the yield of di-DNP-lysine from DNP-lysozyme could be improved by treatment with xanthydrol before hydrolysis, the procedure recommended by Dickman and Asplund was followed (13).

Results

Solubility and Refractive Index Increment

BPA dissolved readily at room temperature in hydrazine and in hydrazine containing 0.05 M NaCl to form 1% solutions. In the presence of 0.2 M NaCl, complete solution required about one hour. In hydrazine hydrate, especially when 0.05 M NaCl was added, BPA required several hours for dispersion.

Lysozyme dissolved easily in salt-free hydrazine but required several days in the presence of 0.1 *M* NaCl.

Two determinations of the refractive index increment of BPA in hydrazine containing 0.05 *M* NaCl gave the values 0.109_8 and 0.109_4 ml/g at 5780 Å and 25° C. The concentration was measured by drying portions of solvent and solution to constant weight in vacuum at 105° C.

Viscosity

Figure 1 shows the viscosity changes with time of 1% BPA solution in hydrazine with and without 0.05 *M* NaCl. The viscosity decreased rapidly at first, to a steady value after about five days. The reduced viscosity (η_{sp}/c), extrapolated to zero time, was 0.8 dl/g in hydrazine containing salt. The comparable value for BPA in aqueous media is 0.042 dl/g (14). The limiting value reached after 5 days in hydrazine was 0.21 dl/g.

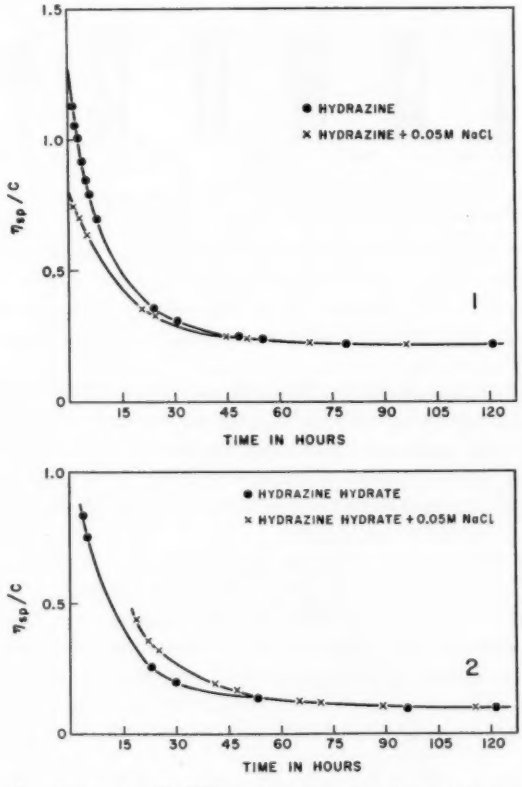

FIG. 1. Viscosity changes in 1% BPA solution in hydrazine and hydrazine containing 0.05 *M* NaCl. Temperature 20° C.

FIG. 2. Viscosity changes in 1% BPA solution in hydrazine hydrate and hydrazine hydrate containing 0.05 *M* NaCl. Temperature 20° C.

Hydrazine hydrate had the similar effect shown in Fig. 2. Because of slow solution, there was a 3-hour delay with the salt-free solution and a 24-hour delay with the salt-containing solution before the first measurements could be made. The limiting value of the reduced viscosity after 5 days was 0.10 dl/g.

Sedimentation Velocity

The sedimentation behavior of BPA in hydrazine containing 0.05 *M* NaCl is shown in Fig. 3. The patterns 3 hours after preparation of the solution are in Fig. 3A and after 7 days at 5° C in Fig. 3B. A single boundary appears in

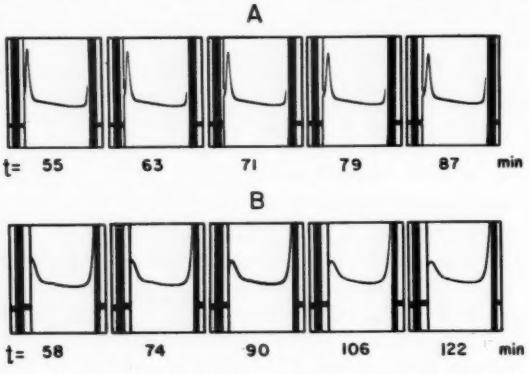

FIG. 3. Sedimentation patterns of BPA in hydrazine containing 0.05 *M* NaCl; (A) 3 hours after preparing solution, (B) after 7 days' storage at 5° C.

both cases but with the longer-stored solution, the boundary separates from the meniscus more slowly and spreads much more rapidly than with the freshly prepared solution. The patterns shown by oxidized BPA in hydrazine with 0.05 *M* NaCl at 5° C and by BPA in hydrazine with 0.2 *M* NaCl at 20° C were similar in appearance.

Table I lists sedimentation coefficients measured for solutions of BPA and oxidized BPA in hydrazine after storage for the times and conditions indicated. The sedimentation coefficient measured in 0.05 *M* NaCl is lower than that measured in 0.2 *M* NaCl (both marked * in Table I), which suggests that the lower salt content is insufficient to eliminate charge effects. The higher salt concentration is probably adequate.

Solutions of BPA in hydrazine hydrate with 0.05 *M* NaCl stored at 5° C gave the sedimentation patterns shown in Fig. 4. Sedimentation coefficients for the boundary which separated from the meniscus are listed in Table II. The patterns obtained soon after solution was complete, Fig. 4A, show a single boundary. After longer storage, however, a second peak of degraded material developed that did not separate from the meniscus (Fig. 4B and C), together with aggregated material whose sedimentation rate increased with storage time.

Molecular Weight

Molecular weight measurements were made on samples of a solution (about 0.8%) of BPA in hydrazine containing 0.2 *M* NaCl and held at 20° C. The

TABLE I

Sedimentation coefficients of bovine plasma albumin in hydrazine

Material	NaCl content, *M*	Protein conc., %	Storage temp., °C	Storage time, hours	$s_{20,w} \times 10^{13}$
BPA	0.05	1.1	5	3	0.84
	0.05	1.0	5	72	0.71
	0.05	0.7	5	72	0.75
	0.05	0.9	5	168	0.52
*	0.05	0.8	20	3	0.79
*	0.2	0.8	20	3	1.21
†	0.2	0.8	20	1	1.71
†	0.2	0.8	20	3	1.08
†	0.2	0.8	20	72	0.68
†	0.2	0.8	20	168	0.61
Oxidized BPA ‡	0.05	1.0	5	1	0.84
‡	0.05	1.0	5	72	0.72
‡	0.05	0.7	5	72	0.80
‡	0.05	1.0	5	192	0.60
§	0.05	1.0	5	72	0.72

*Same protein solution in hydrazine added, after storage, to solid NaCl.
†One solution prepared and sampled serially.
‡Oxidation at −7° C.
§Oxidation at 25° C.

TABLE II

Sedimentation coefficients of faster component of bovine plasma albumin in hydrazine hydrate containing 0.05 *M* NaCl after storage at 5° C

Time after preparation of solution	$s_{20,w} \times 10^{13}$
4 days	2.70
4 days	4.10
16 days	7.5
31 days	11.9

first sample was taken as soon as solution was complete, 1 hour after addition of protein to the hydrazine–salt mixture. Later samples were taken at 3 hours, 3 days, and 7 days. Measurements made at the meniscus ends of the solution columns are plotted in Fig. 5. Average molecular weight values calculated from the slopes of the lines of Fig. 5 are given in Table III and show a decrease, rapid at first and slower later.

TABLE III

Molecular weights of bovine plasma albumin after standing in hydrazine containing 0.2 *M* NaCl at 20° C

Standing period	Molecular weight (from slopes of Fig. 5)
1 hour	57,000
3 hours	35,000
3 days	9,300
7 days	4,500

The deviations of points from the lines drawn in Fig. 5 indicate heterogeneity, especially in the first two samples. The molecular weight values reported are

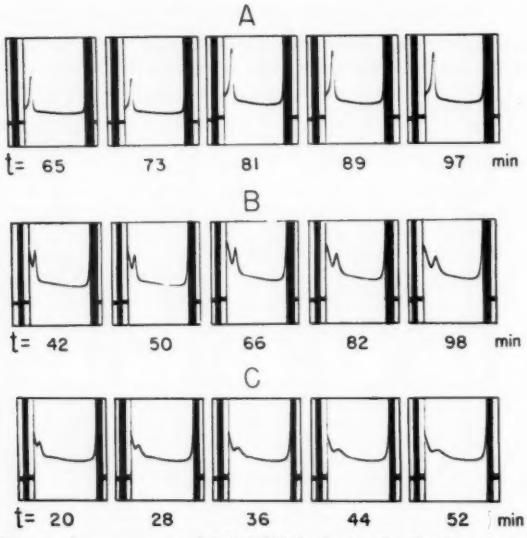

FIG. 4. Sedimentation patterns of 1% BPA in hydrazine hydrate containing 0.05 *M* NaCl; (A) 4 hours after preparing solution, (B) after 4 days' storage, (C) after 16 days' storage at 5° C.

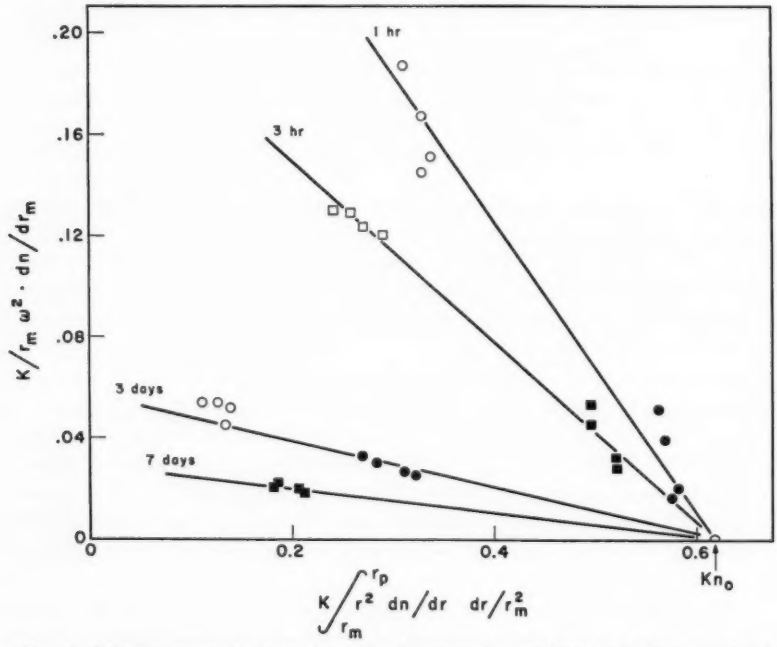

FIG. 5. Measurements made on the region between the meniscus and plateau region in the ultracentrifuge cell. Open symbols 25,980 r.p.m.; closed 42,040 r.p.m.

therefore averages definable only in terms of the experimental conditions. Since the latter were maintained constant except for the standing period, the molecular weights are comparable and show the course of the degradation of the protein.

N-terminal Amino Acid Residues in BPA after Hydrazine Treatment

The ether extract of the acidified reaction mixture (extract I) always contained a trace of DNP-aspartic acid and the amount increased on longer exposure to hydrazine. This may be another example of a hydrolytic removal of N-terminal DNP-aspartic acid under the very mild alkaline conditions of the FDNB reaction (4). In the ether extract of the hydrolyzate of the DNP-protein (extract II) aspartic acid was the only amino acid found with native BPA and after 1-hour treatment with hydrazine. After 6 hours' treatment, the DNP-derivatives of aspartic and glutamic acids, serine, glycine, histidine, threonine, alanine, lysine, leucine, and valine were present and their amounts increased and phenylalanine appeared on further treatment. Figure 6 illustrates

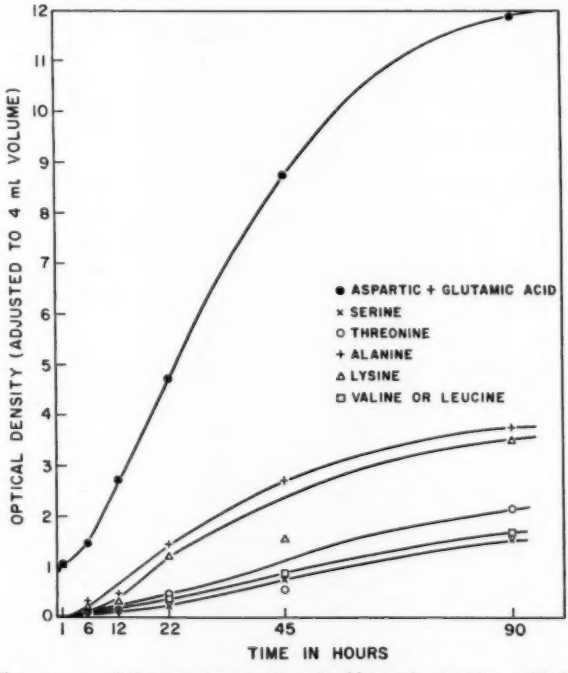

FIG. 6. Time course of the appearance of major N-terminal amino acids in hydrazine-treated bovine plasma albumin. Ordinate, optical density adjusted to 4 ml of solution of DNP-amino acid recovered from chromatography of ethereal extract of hydrolyzate of DNP-protein.

the time course of appearance of the major amino acids excepting glycine and histidine, which were not estimated with an accuracy comparable to the other

amino acids. There appears to be a decrease in the rate of appearance after about 45 hours' exposure to hydrazine at 20° C.

A sample of BPA stored for 45 hours at 20° C in hydrazine hydrate was examined and the same general pattern found although there were quantitative differences from that found with hydrazine (Table IV). Aspartic and glutamic acids were lower in amount; serine, threonine, phenylalanine, and valine plus leucine were higher than in hydrazine treatment.

TABLE IV

DNP-amino acids recovered from hydrolyzates of 50 mg bovine plasma albumin treated with hydrazine or hydrazine hydrate for 45 hours and subsequently reacted with fluorodinitrobenzene

	Optical density adjusted to 4 ml	
DNP-amino acid	Hydrazine	Hydrazine hydrate
Aspartic acid	7.16	4.8
Glutamic acid	1.61	1.08
Serine	0.75	1.44
Glycine	*	*
Histidine	*	*
Threonine	0.55	2.00
Alanine	2.70	2.20
Lysine	1.54	*
Valine + leucines	0.88	1.68
Phenylalanine	0.31	0.90

*Present but not quantitatively assessed.

Oxidized BPA after 45 hours' treatment with hydrazine or hydrazine hydrate (Table V) showed a similar appearance of new N-terminal amino acids. Except that di-DNP-histidine was absent in both treatments of oxidized BPA, the same pattern was observed as with BPA, including the quantitative differences between hydrazine and hydrazine hydrate treatments.

TABLE V

DNP-amino acids recovered from hydrolyzate of 50-mg samples oxidized bovine plasma albumin treated with hydrazine or hydrazine hydrate for 45 hours and subsequently reacted with fluorodinitrobenzene

	Optical density adjusted to 4 ml	
DNP-amino acid	Hydrazine	Hydrazine hydrate
Aspartic acid	6.29	4.68
Glutamic acid	1.46	1.78
Serine	1.10	2.36
Glycine	*	*
Histidine	0	0
Threonine	0.99	2.48
Alanine	2.42	1.96
Lysine	1.96	*
Valine + leucines	0.80	2.46
Phenylalanine	0.46	0.99

0 Not present.
*Present, but not quantitatively assessed.

No DNP-arginine or DNP-cysteic acid was present in any of the aqueous phases.

Effect of Hydrazine Treatment on Lysozyme

Table VI gives the results of treatment of lysozyme with hydrazine. Besides the N-terminal lysine of the protein, prolonged treatment at room temperature

TABLE VI

DNP-amino acids recovered from hydrolyzates of 10-mg samples of lysozyme treated with hydrazine and subsequently reacted with fluorodinitrobenzene

DNP-amino acid	Optical density of DNP-amino acid adjusted to 4 ml			
	Without xanthydrol		With xanthydrol	
	No hydrazine	48 hr hydrazine	No hydrazine	68 hr hydrazine
Lysine	0.16	0.18	0.14	0.21
Aspartic acid	0	0.11	0	0.04
Serine	0	*	0	0.02
Threonine	0	0.12	0	0.04
Alanine	0	0.07	0	0.07

0 Not present.
*Present in small amount.

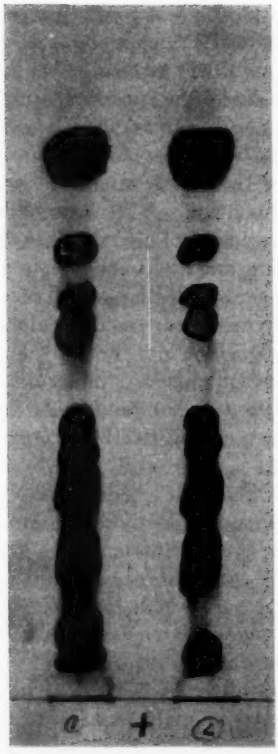

FIG. 7. High voltage paper electrophoresis of hydrazine-degraded lysozyme; 35 volts/cm, 1 hour, in pyridine–acetic acid buffer, pH 6.3. No. 1, load about 2.5 mg; No. 2, about 1 mg. Pattern developed with ninhydrin.

led to the appearance of aspartic acid, serine, threonine, and alanine in N-terminal positions. Trace amounts of some other unidentified DNP-amino acids were also present after 48 hours. The low yield of the N-terminal lysine is in accord with the findings of others (15). Prior treatment of the protein with xanthydrol had little effect on the yield of di-DNP-lysine.

High voltage paper electrophoresis of the product of 3 days' treatment of lysozyme with hydrazine at 20° C showed (Fig. 7) at least 10 separate components.

Discussion and Conclusions

The physical and chemical measurements reported here show that hydrazine and hydrazine hydrate, even at low temperatures, extensively degrade the two proteins tested. This action is probably general and in consequence, neither compound is likely to be a satisfactory solvent for proteins. Bradbury (16) in a study of the kinetics of the hydrazinolysis of peptides found a wide range of reactivity of peptide bonds towards hydrazine, and calculations indicated that some would rupture at moderate and low temperatures. He predicted that these bonds would be sensitive in proteins as well.

The action of hydrazine on BPA appears to be twofold. First and almost immediately, an opening up of the molecule is shown by the high viscosity of the freshly prepared solution and the lowered sedimentation rate. Reduction of disulphide bonds by hydrazine (17) and rupture of intramolecular hydrogen bonds would unfold the polypeptide chains into an extended configuration. Rees and Singer (1) noted that BPA after brief treatment in hydrazine had become insoluble in aqueous media. The second action of hydrazine is a slow degradation to smaller particles. This is shown by the decreasing solution viscosity, sedimentation rate, and molecular weight as well as by the appearance of new N-terminal amino acids. We have also observed that the products of prolonged treatment with hydrazine are readily soluble in water. The degraded material is doubtless heterogenous, and though the molecular weights reported in Table III are not the weight average molecular weights of the respective preparations, since the meniscus region tends to be deficient in larger molecules, they show that the product of degradation consists predominantly of large peptides.

The absence of some amino acid residues as N-terminal and the lack of correlation of those that do appear with the amino acid content of BPA (18) indicates that the breakage of the protein chains is not random.

The appearance of new N-terminal residues in lysozyme (Table VI) also has no close connection with the amino acid composition of this protein (18). The discreet spots shown after paper electrophoresis (Fig. 7) of hydrazine-degraded lysozyme also indicate non-random breakage of the polypeptide chain and suggest usefulness of partial hydrazinolysis in amino acid sequence studies.

The over-all effect of hydrazine hydrate on BPA resembles that of hydrazine although there are differences in detail. The lower value of the reduced viscosity approached after several days, 0.10 dl/g compared to 0.21 dl/g in hydrazine, may reflect differences in extent of breakdown or different effects of the medium

on shape. The different patterns of appearance of new N-terminal amino acids found with both oxidized and unoxidized BPA (Table IV, and V) after hydrazine and hydrazine hydrate treatments show that the susceptibilities of peptide bonds in these proteins towards these two reagents are not the same. Also the different appearance of the sedimentation diagrams (compare Figs. 3 and 4) show that hydrazine hydrate has an effect different from that of hydrazine. The sedimentation rate of the single boundary in freshly prepared hydrazine hydrate solution (Table II) is much higher than the comparable rate in hydrazine (Table I). Also the appearance of aggregated material with a sedimentation rate increasing with time as it decreases in amount has no counterpart in hydrazine solution. This aggregate is still discernible after 1 month at 5° C.

No effect of treatment of BPA with performic acid before solution in hydrazine appears in the sedimentation behavior. The non-appearance of histidine among the N-terminal residues of hydrazine treated oxidized BPA is the major effect of oxidation, suggesting that the lability of this histidine peptide bond toward hydrazine is greatly reduced by the conversion of cysteine to cysteic acid.

Both hydrazine and hydrazine hydrate at low temperature have a degradative effect on bovine plasma albumin and lysozyme. The appearance of new N-terminal amino acids indicates that the predominant degradative action is hydrazinolysis of susceptible peptide bonds. There is also an initial rapid action which is probably an opening up of the native compact configuration of the protein and could arise from reduction of disulphide linkages and rupture of intramolecular hydrogen bonding. The production of a mixture of large peptides may have application in amino acid sequence studies (16).

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THE IN VITRO METABOLISM OF ERYTHROCYTES FROM NEWBORN INFANTS[1]

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With the technical assistance of ANN FAIRBAIRN

Abstract

In the red blood cells of the newborn there is a rapid fall in non-hydrolyzable phosphate during in vitro incubation. This difference appears to be due to a decreased rate of synthesis of 2,3-diphosphoglyceric acid in the erythrocyte of the newborn. In addition the incorporation of P^{32} orthophosphate into the red blood cell is slower in the newborn than in the adult. During 4° C storage of blood of adults and newborns there is a progressive fall in phosphate esters which is similar in both groups.

The erythrocytes of the newborn contain more potassium and water than those of adults. During storage at 4° C the cells of the newborn lose potassium more rapidly than those of the adult. This may be related to differences in membrane permeability.

There are numerous structural and metabolic differences between the erythrocytes of the newborn and those of the adult. The metabolic differences in newborns include the inability to reduce methemoglobin as rapidly as adult cells (1) and the rapid acceleration of glycolysis in the presence of synthetic vitamin K (2).

The red blood cell is thought to obtain its energy primarily from the breakdown of glucose to lactic acid through a series of phosphate ester intermediates (glycolysis). A study of these phosphate esters provides some information regarding the production of energy within the erythrocyte.

Erythrocytes stored at 4° C undergo a number of metabolic changes which are collectively referred to as "the lesion of storage". This is characterized by a progressive fall in glycolytic rate, a decrease in the phosphate ester intermediates of glycolysis, a fall in potassium, and a rise in sodium within the red cell (3).

The effect of 4° C storage on cord blood has been studied previously. In 1954 Sjolin studied the behavior of cord and adult blood during storage in glucose and heparin at 4° C and 37° C (4). He found that, following collection, adult cells hemolyzed less rapidly and lost potassium at a slower rate than cord blood erythrocytes. Greenwalt and Ayers measured the phosphate partition of cord blood after 21 days' storage in acid–citrate–dextrose solution and found a greater rise in inorganic phosphate concentration in the cord erythrocytes than in adult erythrocytes (5).

This paper describes further in vitro studies on these two groups of cells with particular reference to the differences in potassium exchange and phosphate ester levels and the effect of 4° C storage on these aspects of erythrocyte metabolism.

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Materials and Methods

Cord blood was obtained by syringe from the surface veins of the placenta immediately after birth. Venous blood was obtained from infants and adults. Heparin (100 units per 10 ml blood) was used as anticoagulant. When buffer was used as the incubation medium the cells were first washed twice in buffer and then suspended in sufficient buffer to restore the original volume.

BUFFER

Sodium veronal Sodium acetate	*M*/7 *M*/7	20 ml
N/10 HCl		16 ml
8.5% saline		8 ml
5.5% glucose		3 ml
Na_2HPO_4	*M*/15	.825 ml
KH_2PO_4	*M*/15	.175 ml
H_2O to pH = 7.45		100 ml

In the storage experiments all samples were collected in acid–citrate–dextrose solution.*

All phosphate determinations were carried out on whole blood. Phosphate was estimated by the method of Fiske and Subbarow (6). Total acid soluble phosphorus (TASP) was determined by digesting the sample in 10 *N* H_2SO_4 and therefore included both inorganic and organic phosphorus. The organic acid soluble phosphorus (OASP) was subdivided depending on the ease of hydrolysis in 1 *N* HCl at 100° C. The easily hydrolyzable phosphorus and the difficultly hydrolyzable phosphorus fractions refer to those esters hydrolyzed by 1 *N* H_2SO_4 at 100° C during 7 and 100 minutes respectively. The phosphorus esters not hydrolyzed during 100 minutes are referred to as the non-hydrolyzable fraction (NH). In the erythrocytes of adults these fractions are thought to consist of specific compounds (7). The easily hydrolyzable phosphorus represents the two terminal phosphates of ATP, the terminal phosphate of ADP, and perhaps other high energy phosphates. The non-hydrolyzable phosphorus of fresh erythrocytes consists mostly of 2,3-diphosphoglyceric acid. The difficultly hydrolyzable phosphorus fraction represents most of the other phosphate esters (hexose phosphates, etc.). The organic phosphorus of whole blood is almost entirely found in the red blood cell (7); therefore in this study the values for these compounds are expressed as mg phosphorus/100 ml RBC.

The measurement of phosphate uptake was carried out with radioactive phosphorus as follows: 0.2 microcuries of P^{32} (as sodium phosphate) was added to 10 ml of blood. The mixture was allowed to incubate for 20 minutes at 37° C, following which samples were taken at 30-minute intervals for 2 hours. A 0.5-ml portion of plasma was placed in a counting crucible with 1.5 ml of water and counted in a Geiger–Muller end-tube counter. The uptake of phosphate by the erythrocyte was calculated from the disappearance of P^{32} from the plasma.

Glucose was determined by the Nelson modification of the Somogyi method (8).

*(N.I.H. solution B).

Hematocrits were determined by the method of Guest and Siler (9). The hematocrits obtained with this method were similar to those using a Wintrobe hematocrit tube. The observed hematocrits were corrected for trapped plasma by multiplying by .975, a figure obtained from Chaplin and Mollison's studies using Wintrobe tubes (10).

Sodium and potassium were estimated by flame photometry. Red blood cell potassium values were calculated from the whole blood and plasma potassium using the corrected hematocrit as previously described.

Red cell sodium values were determined as follows: Whole blood was centrifuged at 1500 r.p.m. for 10 minutes in a 15-ml centrifuge tube. The plasma was removed and its sodium concentration determined. The remaining red cell mass was transferred by pipette to fill a glass tube 100 mm × 3 mm (the approximate size of the standard Wintrobe hematocrit tube). This was centrifuged at 3000 r.p.m. at a radius of 15 cm for 55 minutes. The tube was then broken neatly at three-quarters of its length, well below the top of the red cell column. The contents of the tube were washed out with water using a long V-shaped No. 18 needle and a 5-ml syringe. The washings were analyzed directly in a flame photometer or, if too thick, were first ashed. The amount of trapped plasma in these tubes was estimated in 12 specimens using plasma labelled with Evans blue and found to range from 1.91% to 2.42% with a mean of 2.16%. To measure the volume of red cells used the tube was cleaned, dried, and filled from a microburette. The cell volume used was equivalent to the amount of water delivered from the burette, less 2% for trapped plasma.

The water content of whole blood was determined by weighing 1-ml samples before and after drying. The red cell water content was calculated from the whole blood value and the hematocrit, assuming plasma water to be 92% (11). The red cell water was expressed as grams of water/100 g red cells.

Procedures and Results

A. Changes in Phosphate Esters during Incubation

1. Incubation for 5½ Hours at 37° C

The phosphate esters of whole blood were analyzed before and after incubation at 37° C. Sufficient glucose was added to each specimen to bring the blood glucose to 200–300 mg%. Hematocrits and phosphate analyses were done on whole blood at zero hours and after 5½ hours' incubation.

Table I shows the values of erythrocyte phosphate esters in 12 adults and 8 newborns. Whereas there is no significant difference between adult and placental blood at zero hours, there is a marked difference on incubation. In placental blood the mean fall in OASP during 5½ hours' incubation was 13.0 mg/100 ml RBC (S.D. ± 6.5) whereas in the adult blood the fall was 2.4 mg/100 ml RBC (S.D. ± 1.6). This difference is highly significant ($p < .001$).

The observed fall in phosphate esters appears to be largely due to a decrease in the non-hydrolyzable phosphorus. In the adult group the decrease in this fraction was 2.1 mg/100 ml RBC (S.D. ± 3.1), in the newborn the fall was 12.3 mg/100 ml RBC (S.D. ± 6.6). This difference is highly significant ($p < .001$). There was no significant difference in the easily hydrolyzable and

TABLE I

Erythrocyte phosphate ester values in adults and newborn infants before and after 5½ hours' incubation at 37° C

Incubation period	Adult ($n=12$)				Newborn infant ($n=8$)			
	Organic acid soluble phosphorus, mg/100 ml RBC				Organic acid soluble phosphorus, mg/100 ml RBC			
	Total	E.H.*	D.H.	N.H.	Total	E.H.	D.H.	N.H.
0 hour	45 ±7.6	5.9 ±2.7	4.5 ±5.2	34 ±6.1	49 ±5.0	7.1 ±1.2	6.0 ±2.9	36.0 ±3.2
5½ hour	43 ±6.8	6.2 ±1.65	4.8 ±1.1	32 ±5.8	36 ±4.2	6.6 ±2.9	5.4 ±1.95	23.7 ±4.4
Change	−2.4 ±1.6	+ .2 ±1.83	+ .2 ±1.5	−2.1 ±3.1	−13.0 ±6.5	− .5 ±2.6	− .65 ±2.0	−12.3 ±6.6

NOTE: Each value is the mean of all experiments ± 1 standard deviation.
*E.H. Easily hydrolyzable phosphorus compounds.
D.H. Difficultly hydrolyzable phosphorus compounds.
N.H. Non-hydrolyzable phosphorus compounds.

difficultly hydrolyzable phosphorus in the adult and newborn groups before or after incubation.

In another series of 23 adults and 16 newborns glucose was not added to the blood prior to incubation. The mean fall of OASP in the adults was 1.73 mg/100 ml RBC (S.D. ± 1.76) whereas in newborns it was 15.0 mg/100 ml RBC (S.D. ± 6.41) (Fig. 1). These differences are highly significant ($p < .001$). To prove that the changes during incubation were a property of the red cells and not the plasma, experiments were carried out in which the red cells of the newborn were incubated in buffer or in adult plasma. In each of these experiments the changes were similar to those found when plasma of newborns was used.

The erythrocytes of children older than 2 years resembled those of the adults in that the changes in phosphate esters during incubation were slight. Up to 2 years of age there was considerable variation; both the newborn and adult pattern of phosphate changes were found throughout this period (Fig. 2).

2. Effect of Iodoacetic Acid on Phosphate Ester Changes during 5½ Hours' Incubation at 37° C

The blood of five adults and four newborns was incubated at 37° C for 5½ hours in the presence of 10^{-3} *M* iodoacetic acid. This quantity was sufficient to completely inhibit glycolysis. The results of this experiment are illustrated in Fig. 1. The mean fall in the adult group in the presence of iodoacetic acid increased from 1.7 mg/100 ml RBC to 20.0 mg/100 ml RBC. In the newborn the mean fall increased from 15.0 mg/100 ml RBC to 20.4 mg/100 ml RBC. It will be noted that in the presence of iodoacetic acid the mean decrease in OASP during incubation became the same in both groups. The fall in non-hydrolyzable phosphorus which occurs in adult cells incubated with 10^{-3} *M* iodoacetic acid was studied in 10 samples of blood and was found to have a mean of 21.3 mg/100 ml RBC.

3. Effect of Adenosine on the Changes in Phosphate Esters during 5½ Hours' Incubation at 37° C

Adenosine (6.25 mmoles/100 ml RBC) was added to the whole blood of three adults and five newborns and incubated for 5½ hours at 37° C. The results,

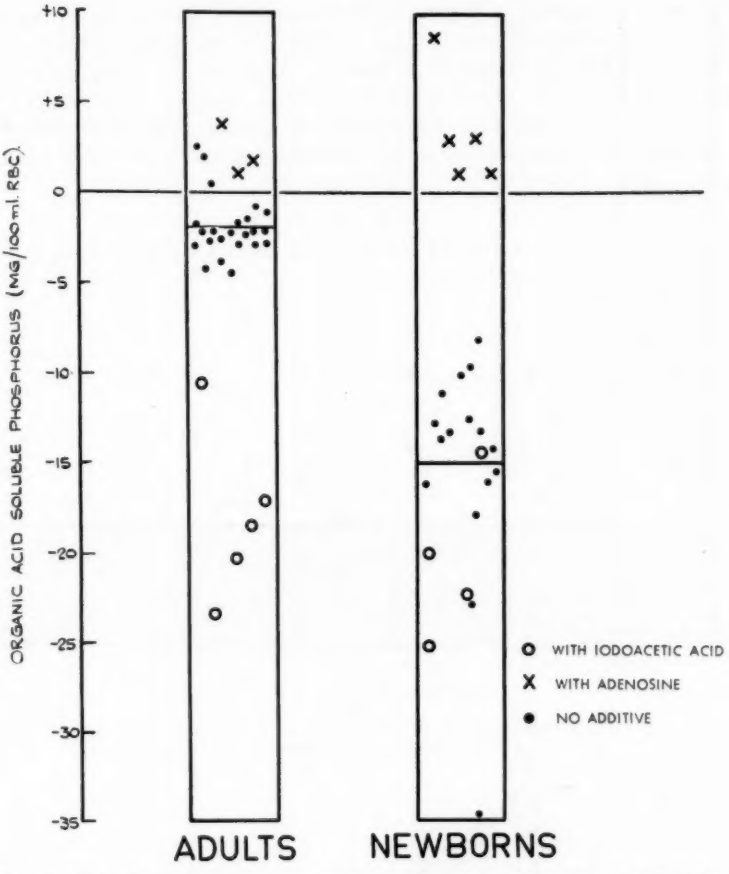

FIG. 1. The change in erythrocyte organic acid-soluble phosphorus during 5½ hours' incubation at 37° C. Whole blood was incubated for 5½ hours' at 37° C with: (*a*) no additive; (*b*) iodoacetic acid to a final concentration of 10^{-3} molar; (*c*) adenosine to a final concentration of 6.25 mmole/100 RBC.

shown in Fig. 1, indicate that there is an increase in OASP during incubation in both groups. In five newborns the mean rise was 3.4 mg%, whereas, in the three adults the rise was 1.7 mg%.

B. The Entry of P^{32} *Orthophosphate into the Erythrocyte*

The rate at which P^{32} leaves the plasma to enter the red cell was studied in seven adults and five newborns. The rate of phosphate entry was more rapid in the adult blood than in the blood of newborns. Thus, the mean rate constant for the fall in plasma radioactivity of the adult group was .127 (S.D. ± .016), and that of the newborn group was .175 (S.D. ± .021) (Fig. 3). These differences are highly significant ($p < .001$).

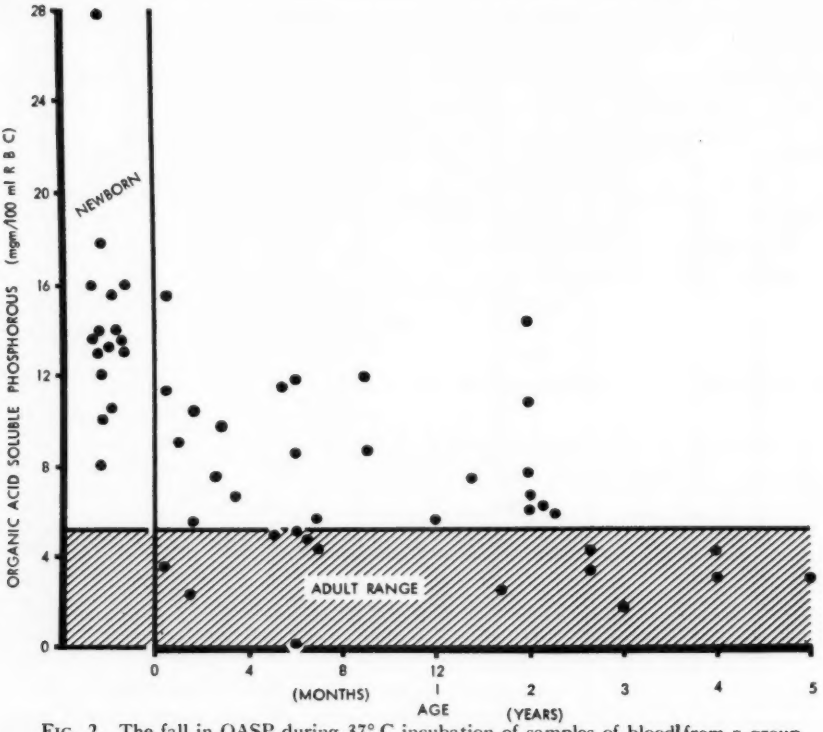

FIG. 2. The fall in OASP during 37° C incubation of samples of blood from a group of children, 0–5 years of age. The shaded area represents the range observed in samples of blood from normal adults.

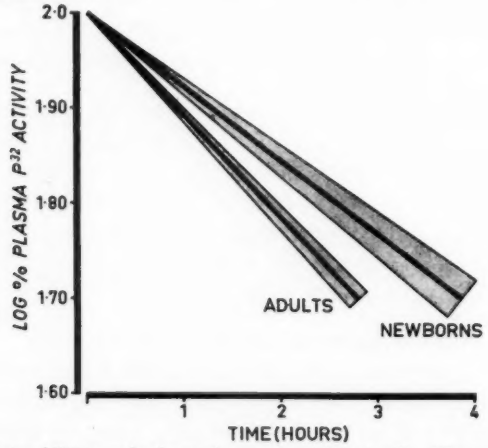

FIG. 3. The rate of P^{32} transfer from plasma to red blood cells. The mean rate constant for the decrease in plasma radioactivity is .237 (S.D. ± .016) for seven adults and .175 (S.D. ± .021) for five newborns. The shaded areas represent 1 standard deviation from the mean.

C. Changes in Erythrocyte Phosphate Esters during 4° C Storage

Table II shows the values for OASP at 0, 7, 14, and 28 days' storage at 4° C in a series of adults and newborns. There is no significant difference in the values for newborn cells as compared to adult cells. Similarly when non-hydrolyzable phosphorus values were examined (Table III) there was again no statistical difference in changes occurring in each group, during the period of storage.

TABLE II

Red cell organic acid soluble phosphorus (mg/100 ml RBC) during storage at 4° C

Day	0	7	14	28
Adult				
Number	4	4	5	4
Mean	42.0	30.4	23.1	5.9
S.D.	±4.1	±7.5	±6.0	±2.3
Cord				
Number	10	9	9	7
Mean	43.4	27.9	17.2	9.8
S.D.	5.0	5.5	2.4	6.7
Significance				
p	>.1	>.1	<.05	>.1

TABLE III

Red cell non-hydrolyzable phosphorus (mg/100 ml RBC) during storage at 4° C

Day	0	7	14	28
Adult				
Number	3	3	3	3
Mean	32.1	16.8	14.2	4.5
S.D.	±1.65	±6.4	±6.6	±4.0
Cord				
Number	7	6	7	6
Mean	33.2	16.	11.6	4.0
S.D.	2.8	7.8	8.0	4.05
Significance				
p	>.1	>.1	>.1	>.1

D. Glucose Utilization

Samples of whole blood of 9 adults and 10 newborns were centrifuged and the plasma and buffy coat removed. The red cells were washed twice with buffer and then resuspended in buffer. Blood glucose was determined before and after 3 hours' incubation. Under these conditions the blood of adults utilized glucose at a rate of 21 mg/100 ml RBC/hour (S.D. ± 7.8) and in the newborn 25.3 mg/100 ml RBC/hour (S.D. ± 6.4). There is no significant difference between these rates.

E. Sodium and Potassium Metabolism in Adult and Cord Erythrocytes

1. Sodium, Potassium, and Water Content of Adult and Cord Erythrocytes in Heparin

These are set forth in Table IV. The potassium content of cord cells is significantly higher than that of cells of adults so that the total potassium plus

sodium is higher in cord cells. Since the red cell water content is also elevated in cord blood there is no significant difference in the total sodium plus potassium of cord and adult cells when expressed as milliequivalents per liter of cell water.

TABLE IV

Erythrocyte sodium, potassium, and water content in adults and newborns

	Water, % (w/w)	Potassium, meq/l. cells	Sodium, meq/l. cells	Sodium and potassium, meq/l. cells
Adult				
Number	17	13	11	10
Mean	63.8	101.3	12.45	114.2
S.D.	±2.43	±4.9	±2.2	±5.2
Newborn				
Number	10	11	11	11
Mean	66.5	106.2	13.7	120.3
S.D.	2.6	5.43	3.24	6.2
Significance p	$<.02$	$<.05$	$>.10$	$<.05$

2. Erythrocyte Sodium and Potassium Changes during Storage at 4° C

Figure 4 shows that during 4° C storage the fall in potassium and rise in sodium of cord cells is more rapid than that of adult cells. In a series of five adults and five newborns the mean rise in erythrocyte sodium during the first

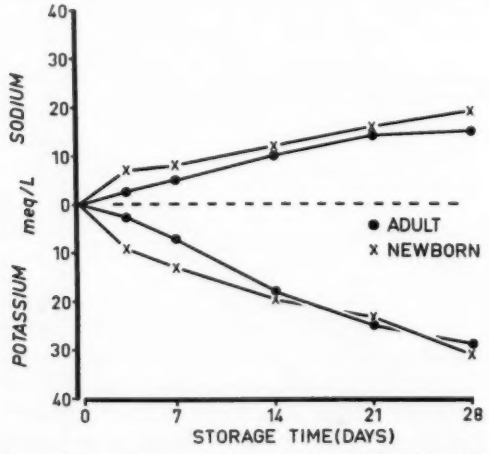

FIG. 4. The changes in erythrocyte potassium and sodium during 4° C storage of adult and newborn blood.

3 days of storage was 2.0 meq/l. (±1.2) and 6.7 meq/l. (±3.8) respectively. These differences are statistically significant ($p < .05$). The total change in red cell sodium during 4 weeks of storage was not significantly different in these two groups.

During 4° C storage the red cell potassium in five adults and five newborns fell 2.8 ± 1.2 meq/l. and 8.3 ± 4.0 meq/l. respectively on the first 3 days. These values are significantly different ($p < .05$). The total fall in erythrocyte potassium at the end of the 4-week period was the same in both groups.

3. Diffusion of Potassium from Hemolyzates of Adult and Cord Blood

To determine if the rapid loss of potassium from fetal red cells was due to a difference in potassium binding capacity of the intracellular components of the two groups of cells the following experiment was performed.

Blood collected in ACD solution or heparin was centrifuged and the plasma and buffy coat removed. The cells were hemolyzed by repeated freezing and thawing until a smear of the hemolyzate examined microscopically showed no intact cells. Five-milliliter samples of the hemolyzates were dialyzed against 1.5 liters of 0.9% (w/v) NaCl at 37° C. Diffusion was allowed to proceed for 30 minutes. Five-milliliter quantities of KCl solution (10–100 meq/l.) were also dialyzed in the same manner. Potassium concentrations of the dialyzate were determined at 10- or 15-minute intervals. It was found that diffusion was a first-order process and a rate constant was calculated.

From Table V it may be seen that hemoglobin solutions from adult and cord blood do not differ in their capacity to bind potassium. The mean diffusion rates from the two series are almost identical: .0229 min^{-1} from adult blood;

TABLE V

Potassium diffusion rates from hemolyzates and (KCl solutions) at 37° C

	Solution, KCl	Hemolyzate	
		Adult	Cord
No. of samples	6	7	7
Mean	.0528	.0229	.0222
S.D.	± .0017	± .0030	± .0071

NOTE: Rate constants are expressed as minutes^{-1}.

and .0222 min^{-1} for cord blood. It should be noted also that diffusion from hemolyzates is markedly slower than from a KCl solution (.0528). The difference is significant ($p < .01$).

4. The Rate of Potassium Uptake

To compare the rate at which potassium can enter the adult and cord red cells, rates were calculated using radioactive potassium (K^{42}). Twenty milliliters of washed erythrocytes, suspended in buffer, were incubated at 37° C for 20 minutes with 0.2 microcuries of K^{42} (as KCl). Samples were then taken at $\frac{1}{2}$-hour intervals and radioactivity of the buffer was measured. The rate constant (Rc) of potassium disappearance from the buffer was then calculated. The potassium uptake equalled

$$(K_1) \times \frac{100 - \text{hematocrit}}{\text{hematocrit}} \times Rc$$

where (K_1) represents the mean concentration of potassium during the incubation period.

The rate of potassium influx is related to the external potassium concentration at low potassium concentrations (12). Thus, the potassium uptake expressed as meq K per liter of cells per hour was plotted against the external potassium concentration. Figure 5 shows that the rate of uptake of potassium in fetal cells is not significantly different from that of adult cells. When potassium uptake rates were expressed as a percentage of the total red cell potassium per hour and then plotted against external potassium concentration, there was again no significant difference in the two groups.

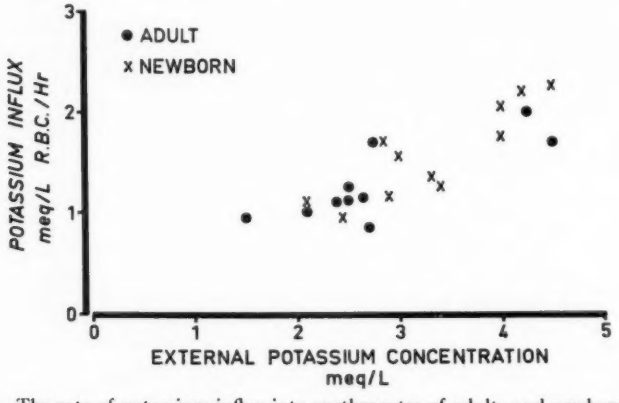

FIG. 5. The rate of potassium influx into erythrocytes of adults and newborn infants. Whole blood was incubated at 37° C for 2 hours in buffer (pH 7.4), containing K^{42}. The rate of potassium influx was calculated from the rate of disappearance of plasma radioactivity over a 2-hour period.

Discussion

A. Phosphate Ester Metabolism in Cord Erythrocytes

When blood of the newborn infant is incubated at 37° C there is a fall in whole blood OASP which is significantly greater than that noted with adult erythrocytes (Table I). The fall appears to be due largely to a decrease in the non-hydrolyzable phosphorus fraction. There is no significant change in the easily hydrolyzable or difficultly hydrolyzable phosphorus fractions during incubation in either adult or cord blood.

In the erythrocytes of the adult non-hydrolyzable phosphorus is thought to represent, almost entirely, 2,3-diphosphoglyceric acid (7). That the non-hydrolyzable phosphorus of fetal cells represents the same compound is suggested by the finding of almost identical levels of non-hydrolyzable phosphorus in both types of cell. In our series this fraction constituted 77% and 76% of the red cell organic acid soluble phosphorus in newborns and adults respectively. The only explanation which has been suggested for the singularly high concentrations of this compound in red cells is that it may be related to the low concentration of glycerate 2,3-diphosphatase within the cell (13).

The fall in non-hydrolyzable phosphorus (presumably 2,3-diphosphoglyceric acid) during incubation of the erythrocyte of the newborn could be due either

to a diminished rate of synthesis or to an increased rate of breakdown or conversion to acid-insoluble compounds. To examine these possibilities the rate of fall of phosphate esters was studied when synthesis was reduced by inhibiting glycolysis with 10^{-3} *M* iodoacetic acid. In adult cells inhibition of phosphate ester synthesis resulted in a very great fall in OASP particularly in non-hydrolyzable phosphorus. Under these conditions the fall in OASP was of similar magnitude in the erythrocytes of both adults and newborns (Fig. 1). These findings suggest that the excessive fall of phosphate esters during incubation of newborn red cells was due to a decreased rate of synthesis. Further evidence suggesting that erythrocytes of the newborn synthesize phosphate esters more slowly than those of the adult in vitro is provided by the studies of the incorporation of inorganic phosphate into the red blood cell. The uptake of P^{32} orthophosphate into the red blood cell is considered to involve incorporation of the phosphate directly into phosphate esters (14). The fact that phosphate incorporation into erythrocytes of the newborn was significantly slower than that of the adult therefore provides further evidence that in vitro the erythrocytes of the newborn are unable to form certain phosphate esters as rapidly as do erythrocytes of adults.

Adenosine is thought to enter the red blood cell, undergo phosphorylysis, and form ribose-1-phosphate (3). This compound is then metabolized by means of the pentose phosphate pathway entering the anaerobic glycolytic cycle at or above the glyceraldehyde-3-phosphate level. As shown in Figure 1, the addition of this compound to erythrocytes of the newborn prevents the characteristic fall in phosphate esters during incubation. This suggests that adenosine may provide an alternate pathway for the synthesis of 2,3-diphosphoglyceric acid in the newborn erythrocyte, possibly by-passing the early steps of glycolysis preceding the formation of glyceraldehyde-3-phosphate.

It therefore appears that during in vitro incubation of cells of the newborn at 37° C there is both a slower synthesis of 2,3-diphosphoglyceric acid and a decreased rate of incorporation of P^{32} orthophosphate, when compared to adult cells. This diminished synthesis of phosphate esters may be due, in part, to a diminution in the activity of the early steps of glycolysis preceding the formation of glyceraldehyde-3-phosphate. However, these changes are not associated with an over-all decrease in the rate of glycolysis of fetal cells since fetal blood utilized glucose at a rate similar to that of adult blood.

Greenwalt *et al.* (5), found a more rapid rise in inorganic phosphorus in cord erythrocytes during storage at 4° C. They also demonstrated differences between adult and cord erythrocytes during 4° C storage in easily hydrolyzable, difficultly hydrolyzable, and non-hydrolyzable phosphorus. We were unable to demonstrate any difference in the rate of fall of OASP and hence rise of inorganic phosphorus in the two groups. Our analyses were carried out on whole blood, in contrast to those of Greenwalt *et al.* (5), which were done on washed red cells. This may explain the differences observed in the inorganic phosphorus values in the two studies. In addition at 4° C there appears to be no difference in the breakdown of 2,3-diphosphoglyceric acid (measured as non-hydrolyzable phosphorus) in these two types of cells whereas at 37° C a

marked difference was observed (Table I). If we postulate that a single step in the syntheses of 2,3-diphosphoglyceric acid is less active in fetal than adult cells (at 37° C) it may be that at 4° C this step is sufficiently depressed in adult cells to abolish the difference. Another possible explanation is that at 4° C the enzyme glycerate 2,3-diphosphatase (present in low concentrations in erythrocytes) may be sufficiently depressed that any rapid breakdown of 2,3-diphosphoglyceric acid in fetal cells is precluded.

B. Sodium and Potassium Metabolism in Cord Erythrocytes

Prior to storage, cord erythrocytes contained an average of 4–5% more water and potassium than adult cells. However, the potassium concentration is similar to that of the adult cell.

The more rapid decrease on storage of intracellular potassium in newborn red cells is in agreement with the findings of Sjolin (4). This is accompanied by a more rapid rise in sodium of these cells. The rate of potassium influx at 37° C did not differ from that of adult cells, nor did potassium diffuse more rapidly from hemolyzates of fetal cells than from hemolyzates of adult cells. These findings suggest that the rapid loss of potassium from fetal cells during 4° C storage may not be related to either a slower rate of uptake of potassium nor to less avid binding of potassium by the intracellular contents of fetal cells. It may be that the observed difference in sodium and potassium exchange between these two types of cells is related to differences in structure of the membrane. An anatomical difference between adult and newborn cells has been shown by electron microscopy (15) by Dervichian *et al.*

Acknowledgments

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THE METABOLISM OF ALDOSTERONE BY SURVIVING DOG AND HUMAN LIVER SLICES[1]

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Abstract

Surviving dog liver slices were incubated with *d,l*-aldosterone-21-monoacetate, *d,l*-aldosterone, *d*-aldosterone, and *d*-aldosterone-21-C^{14}. Human liver slices were incubated with *d,l*-aldosterone-21-monoacetate and *d*-aldosterone. The incubations were performed in a Krebs–Ringer–phosphate medium (pH 7.4), with 200 mg glucose added per 100 ml of medium, at a temperature of 37° C. After incubation, the medium was extracted with chloroform and the crude extract extensively fractionated on column and paper chromatographic systems. In addition to free aldosterone, four metabolic products were isolated, two ring A reduced α-ketolic and two ultraviolet absorbing, non-reducing substances. The partial chemical characterization of these metabolites was attempted. The search for aldosterone metabolites in human urine resulted in the isolation of a substance in acetate form from the urine of a patient suffering from primary aldosteronism which may be identical with one of the ring A reduced metabolites obtained in the in vitro experiments.

Introduction

The metabolism of most of the naturally occurring corticosteroids has been investigated extensively both in vivo and in vitro (21, 42). In contrast, little information is available concerning the metabolic pathways of aldosterone.

It has been established by the use of tritiated aldosterone that normal human adrenals secrete approximately 200 μg of aldosterone daily (7, 55). Only a small portion, 1–5%, can be recovered in urine as unchanged aldosterone (24, 27, 32). The search for urinary metabolites of aldosterone resulted in the isolation of a ring A reduced, α-ketolic compound by Ulick and Lieberman (54, 55).

Investigating the problem by a different approach, Chart and co-workers (18) reported that surviving rat and human liver slices deactivated a crude urinary aldosterone preparation as judged by the loss of sodium retaining activity. Later work has shown that the major in vitro metabolic reactions of aldosterone involve the reduction of the ring A conjugated system, while the reducing side chain is apparently untouched. This has been demonstrated using dog and human liver slices (45) and mouse, rat, and guinea-pig liver homogenates (1). The purpose of the present communication is to describe in some detail investigations related to the metabolism of aldosterone by dog and human liver slices and the isolation and partial characterization of the metabolites formed.

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Methods

Materials

1. *Substrate*

Synthetic *d,l*-aldosterone-21-monoacetate,* *d,l*-aldosterone, and *d*-aldosterone were used.

The synthetic monoacetate was controlled for homogeneity in two paper chromatographic systems (cyclohexane:benzene/propylene glycol (31) and toluene:isooctane (1:1)/70% methanol).

d,l-Aldosterone was prepared from the monoacetate by saponification with $KHCO_3$ – aqueous methanol (51). The product was purified by successive paper chromatography in three systems (37).

Natural *d*-aldosterone was isolated from human late pregnancy urine and purified by successive paper and column chromatography (36, 37).

2. *Medium*

A Krebs–Ringer–phosphate solution (pH 7.4) was used with 200 mg glucose added per 100 ml medium (56). The medium was freshly prepared from stock solutions about 45 minutes before each experiment.

3. *Tissue*

Dog liver samples were obtained from healthy, anesthetized mongrel dogs by partial or total hepatic lobectomy. The tissue was received in ice-cold medium and transported immediately to the laboratory. Slices 0.5 mm thick were cut with a Stadie-Riggs microtome and weighed on a torsion balance. Human liver biopsies were obtained from patients undergoing abdominal surgery. They were received and treated similarly to the dog liver specimens.

Incubation

The substrate was added directly to the medium. The liver tissue slices were transferred to 50-ml beakers containing the medium and substrate (ratio tissue: medium 1:10 w/v). Tissue slices incubated without the steroid and boiled slices incubated with the substrate served as controls.

The incubation proper was performed in a Dubnoff metabolic shaking incubator (Precision Scientific Corp., Chicago, Ill.) for 3–4 hours at $37 \pm 1°$ C, in an atmosphere of oxygen containing 5% CO_2 (rate of flow 3–4 liters/minute).

The incubation was interrupted by immersing the beakers in a mixture of dry ice and acetone. After the contents had frozen completely, the beakers were stored at $-20°$ C until processed.

Extraction

The medium was transferred to a separatory funnel. The tissue was ground up in a mortar with purified sea sand and chloroform. The chloroform extract of the ground tissue was poured into the separatory funnel containing the medium and shaken for 5 minutes. This procedure was repeated six times on each incubation vessel. The solvent portions were pooled, dried over anhydrous sodium sulphate, and evaporated to dryness under reduced pressure. The control vessels were treated in an identical manner.

* Kindly supplied by Dr. K. Thompson, Organon Inc., Orange, N.J., and Dr. Walter Murphy, Ciba Company, Montreal, Que.

Chromatography

The crude chloroform extract was subjected to preliminary purification on a silica gel column (34, 38) using 2.5 g silica gel (200 mesh, Davison).

Eluents: chloroform–acetone 99:1 (v/v), 50 ml and chloroform–acetone 1:1 (v/v), 100 ml. Aldosterone, aldosterone monoacetate, and its metabolites were eluted by the second eluent.

The following paper chromatographic systems were used for isolation of free aldosterone and the metabolic products: toluene/ethylene glycol (39); benzene/55% methanol (11); tertiary butanol–isooctane/water (1:2:1.8) (25). For the isolation of acetates and oxidative degradation products of polar substances the following systems proved to be useful: toluene–isooctane/(1:1) 70% methanol; benzene–cyclohexane (1:1)/propylene glycol (31); isooctane–cyclohexane (1:2)/ethylene glycol.

In all paper chromatographic systems paper strips 60 cm long and 15 cm wide were employed.* All chromatograms were run at room temperature ($25 \pm 2°$ C). For the detection of substances on the chromatographic strips one or more of the following methods were used: (*a*) scanning with ultraviolet light;† (*b*) spraying with alkaline blue tetrazolium (35); (*c*) spraying with alkaline *m*-dinitrobenzene (31); (*d*) spraying with qualitative 2,4-dinitrophenylhydrazine reagent (3). The zones to be investigated were eluted from the chromatographic paper with the aid of a modified Haines device (29), using 95% ethanol‡ as eluent.

For final purification of substances isolated from paper chromatograms, the following methods were used: (*a*) Kieselgur column chromatography (38, 46). This method proved very useful for freeing polar substances from contaminants eluted from the chromatographic paper. Solvents and Kieselgur§ were purified according to Hegedüs *et al.* (30) and Schindler *et al.* (46). Stationary phase: 1 g Kieselgur mixed with 1 ml water. Successive eluents: (1) petroleum ether, 50 ml; (2) petroleum ether:benzene 3:1, 20 ml; (3) petroleum ether:benzene 1:3, 80 ml; (4) benzene, 50 ml.

For the purification of relatively non-polar substances, alumina column chromatography was used (2). A 140×7 mm chromatographic column was filled to a height of 70 mm with activated alumina.‖ Successive eluents were: (1) benzene; (2) benzene – 0.1% ethanol; (3) benzene – 0.5% ethanol; (4) benzene – 2.0% ethanol; (5) 95% ethanol.

Quantitation of Isolated Compounds

(*a*) With compounds absorbing ultraviolet light, absorption curves in 95% ethanol** solution were taken on a Beckman Model DU quartz spectrophoto-

* Whatman No. 2 paper, "especially selected for chromatography". Before use, the paper was washed in a Soxhlet apparatus for 72 hours with a 1:1 v/v mixture of benzene:methanol.

† Mineralight, Model RV 71, Research Equipment Corporation, California. Maximal emission at 254 mμ.

‡ The ethanol for this purpose was twice distilled, the second time from dianisole-bis-4,4′-(3,5-diphenyl)tetrazolium chloride (blue tetrazolium, BT-Dajac) and NaOH pellets.

§ Hyflo Super Cell, Johns Manville.

‖ Activated alumina, Grade: F-20, The Aluminum Co.

** The ethanol for this purpose was twice distilled, the second time from dianisole-bis-4,4′-(3,5-diphenyl)tetrazolium chloride (blue tetrazolium, BT-Dajac) and NaOH pellets.

meter from 220 to 300 mμ. The concentration of the ultraviolet absorbing material was calculated using the following formula:

$$\text{conc. } (\mu\text{g/ml}) = \frac{Mw \times OD_{max} \times 1000}{e}$$

where Mw = molecular weight,
OD_{max} = optical density at maximal absorption,
e = molecular extinction coefficient. (With substances of unknown molecular extinction coefficient, the hypothetical value of 15,800 was used.)

(*b*) With blue tetrazolium reducing substances, the quantitative version of this reaction was used, either in the macro or in the micro modification as described by Nowaczynski *et al.* (35, 37).

Recovery Experiments

Recovery experiments were performed by incubating known amounts of cortisone with deactivated (boiled) liver slices, followed by extraction of the medium and slices and isolation of the steroid. For these experiments cortisone was used rather than aldosterone due to the relative scarcity of the latter substance. Recovery of cortisone after extraction and chromatography on silica gel was 98.4%.

Results

Incubation of Aldosterone with Dog Liver Slices

For these experiments the following forms of the substrate were used: (*a*) *d,l*-aldosterone-21-monoacetate; (*b*) *d,l*-aldosterone, and (*c*) *d*-aldosterone.

The incubation of all three forms of aldosterone yielded two major metabolites in addition to non-metabolized free aldosterone. The two metabolites, designated as metabolite A_1 and metabolite A_2, proved to be α-ketolic and non-ultraviolet-absorbing substances with a rather close resemblance in their physicochemical characteristics. A third substance, metabolite B_1 resulted from the incubation of *d*-aldosterone in addition to metabolite A_1 and A_2. It was not found in incubation mixtures involving synthetic aldosterone.

The incubations were performed under the conditions described earlier. A total of 14.99 mg of *d,l*-aldosterone monoacetate was incubated in the course of five experiments, with a tissue to substrate ratio of about 2000:1. In the case of *d,l*-aldosterone (free) the amount was 4780 μg of substrate with a tissue to substrate ratio of 2000:1, while in the experiment using natural free aldosterone isolated from human pregnancy urine 1260 μg of substrate was incubated with a tissue to *d*-aldosterone ratio of 4400:1.

The amounts of metabolites A_1 and A_2 isolated and the amounts of free aldosterone recovered are shown in Table I. It can be noted from the table that the two metabolites were formed in about equal quantities. There was not much difference in the amounts produced, whether the synthetic aldosterone was free or acetylated; however, *d*-aldosterone gave a fourfold yield of these metabolites. As mentioned earlier, metabolite B was noted only in the experiment involving *d*-aldosterone and was present in small quantities.

A large percentage of the substrate could not be accounted for after the isolation of the metabolic products and the unchanged substrate. It was felt

TABLE I

Different forms of aldosterone incubated with dog liver slices. Quantitative yield of metabolites

Substrate	Metabolites, yield in %			Recovery of free aldosterone, %	Substrate not accounted for, %
	A_1	A_2	B		
d,l-Aldosterone-21-monoacetate	6.1	6.1	—	32.6–58 (41.4)	34–53 (46.5)
d,l-Aldosterone	5.6	6.1	—	21	67.3
d-Aldosterone	21.5	23.4	1.9	10.4	52.8

that this might be due to either of the two following possibilities: (1) the substrate was metabolized to substances which could not be detected by the methods employed; or (2) the metabolites were partly conjugated by the liver tissue. These latter substances, being water-soluble, would not have been extracted with chloroform. No attempts were made to treat the medium by incubation with a β-glucuronidase or sulphatase enzyme preparation or by continuous extraction at pH 1. Similarly no inquiries were made into the nature of the enzymatic reactions involved. These problems will be subject to further studies.

Isolation and Partial Chemical Characterization of the Metabolites (5)

A. Metabolite A_1 and Metabolite A_2

These two substances will be considered together due to the close resemblance in their physicochemical characteristics.

TABLE II

Physicochemical properties of the periodic acid oxidation products of metabolites A_1 and A_2

	Ultraviolet absorption	Blue tetraz. reduction	Spectra in conc. H_2SO_4		Acetylation	Spectra in conc. H_2SO_4 of acetates	
			Max.	Min.		Max.	Min.
Product of A_1	Negative	Negative	265 310	240 285	Acetate formed	270 I 304 410	255 278
Product of A_2	Negative	Negative	312	260	Acetate formed	240 I 272 306 410	257 385

A total of about 1000 μg of each of the metabolites was isolated. However, the products were not accumulated but metabolites isolated from each experiment were treated as unknown compounds and the relative identity of A_1 and A_2 from each incubation was established by the largest possible number of physicochemical characteristics. In this way the reactions to be described were performed several times, but material available for each reaction was limited in most cases to less than 100 μg. This approach excluded the carrying out of such procedures as molecular weight determination or elementary analysis, but it

established the reproducibility of the partial transformation of *d,l*-aldosterone-21-monoacetate, *d,l*-aldosterone, and *d*-aldosterone to metabolite A_1 and A_2 under the incubation conditions described.

The method of isolation and purification of these metabolites is shown in Fig. 1. In a few cases an additional step was included, namely after chromatography on Kieselgur column the metabolites were subjected to high vacuum

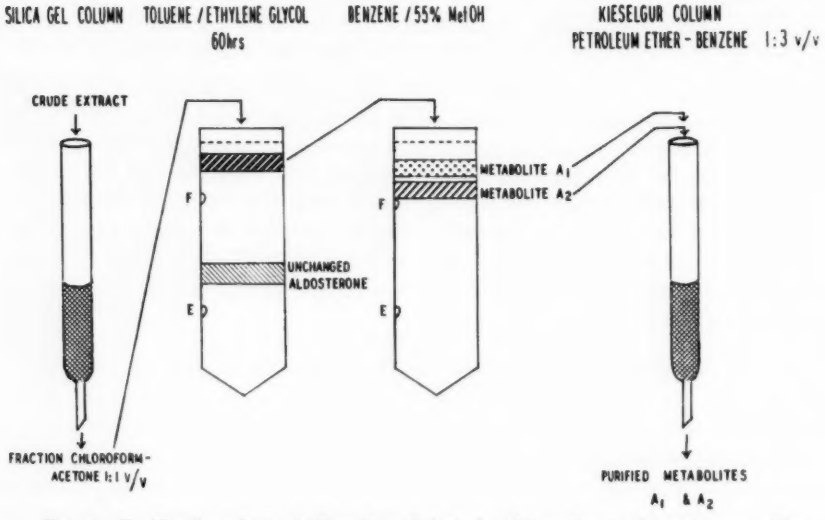

FIG. 1. Purification of metabolites A_1 and A_2 isolated from incubation mixtures. The two metabolites are separately purified on Kieselgur column.

distillation as final step of purification. This proved to be very useful in dealing with samples in the μg range, where crystallization would have been difficult and wasteful.

The metabolites showed the following chemical and physicochemical properties:

1. *Chromatographic Mobilities*

System	Metabolite A_1	Metabolite A_2
Toluene/ethylene glycol	R_{cort}* : 0.22	R_{cort} : 0.22
Benzene/55% methanol	R_{cort} : 0.20	R_{cort} : 0.43
t-Butanol–isooctane/water	R_{THE}†: 0.97	R_{THE}: 1.03

2. *Spectrum in Concentrated Sulphuric Acid* (9, 10)

A_1 Maxima: 312,356 I‡, 410, 570 mμ
Minima: 248,500 mμ

A_2 Maxima: 260 I, 315, 356 I, 410, 570
Minima: 242, 525 mμ

* Mobility relative to cortisone.
† Mobility relative to tetrahydrocortisone.
‡ "I" denotes inflection of plateau.

3. *Spectrum in "100%" Phosphoric Acid* (40, 41)
A_1: Series of inflections at 290, 420, and 515 mμ
A_2: Series of inflections at 290, 340, 422, and 510 mμ

Interpreting their data on the spectra of steroids in concentrated acids, both Bernstein and Lenhard (9, 10) and Nowaczynski and Steyermark (40, 41) did assign a specific absorption range for compounds with Δ^4-3 keto grouping. This range is from 279 to 300 mμ in concentrated sulphuric acid and from 275 to 290 mμ in 100% phosphoric acid. Neither of the two metabolites showed any peak absorption in this region.

4. *Blue Tetrazolium Reduction* (35, 37)
This reduction was positive and instantaneous.

5. *Ultraviolet Absorption*
The results were negative in 95% ethanolic solution (220–300 mμ) and on paper (254 mμ).

6. *Alkaline m-Dinitrobenzene Reaction* (31, 13)
This was negative in solution and on paper.

7. *Acetylation* (51)
Both metabolites formed acetates after treatment with acetic anhydride in pyridine at room temperature for 48 hours. In the paper chromatographic systems tried, the acetates of A_1 and A_2 showed identical mobilities:
Benzene/55% methanol: R_{cort}: 2.33,
Toluene–isooctane/70% MetOH: R_{THEAc}*: 1.17,
Cyclohexane (1): isooctane (2)/ethylene glycol R_{DOCA}†: 1.41.
Both acetates reduced alkaline blue tetrazolium on paper and in solution.

8. *Acetylation* ("mild", according to Mattox *et al.*) (33)
This procedure was devised by Mattox *et al.* for the selective 21-acetylation of aldosterone with the use of diluted reagents and a 1.25-hour reaction time. Both metabolites gave acetates different from the products described under paragraph 7. Both acetates reduced blue tetrazolium and had identical mobilities in the benzene/55% methanol system. (R_{cort}: 1.76).

9. *2,4-Dinitrophenylhydrazone Formation* (3, 28)
Hydrazones were formed by both substances on paper and in solution. In the procedure of Gornall and Macdonald (28), neither metabolite gave a 5-minute reaction characteristic for Δ^4-3 keto grouping. Both formed hydrazones after 90 minutes of heating at 59° C. The hydrazones gave maximal absorption at 510 mμ.

10. *Oxidative Degradation Studies*
Using periodic acid (50).—80 μg of each A_1 and A_2 were oxidized with HIO_4. Both metabolites yielded products different from the starting material and insoluble in saturated sodium bicarbonate. They were purified on an activated alumina column and eluted from that column with benzene – 0.1% ethanol.

* Mobility relative to tetrahydrocortisone–diacetate.
† Mobility relative to desoxycorticosterone–acetate.

The oxidation products had the following physicochemical characteristics (see also Table II):

a. Ultraviolet absorption: negative.

b. Blue tetrazolium reduction: negative.

c. Alkaline *m*-dinitrobenzene reaction: negative in solution, faint blue color on paper.

d. Acetylation (acetic anhydride in pyridine, room temperature for 48 hours): acetate formed.

Using CrO_3 *in glacial acetic acid* (50, 57).—50 μg of each A_1 and A_2 were oxidized. They gave products different from the starting material and different from the HIO_4 oxidation products. They were insoluble in saturated sodium bicarbonate solution. These products had the following physicochemical characteristics:

a. Ultraviolet absorption: negative for both.

b. Blue tetrazolium reduction: negative for both.

c. Alkaline *m*-dinitrobenzene reaction: negative for both in solution. On paper both gave moderately strong blue color.

d. 2,4-Dinitrophenylhydrazine reaction: both reacted on paper.

e. Chromatographic mobilities: the paper system toluene–isooctane/70% methanol oxidized metabolite A_1 (R_f*: 0.72) and oxidized metabolite A_2 (R_f: 0.81).

11. *Infrared Spectrum*

The infrared spectrum of metabolite A_1 and A_2 (purified by paper chromatography, Kieselgur column, and high vacuum distillation) was kindly taken by Dr. R. Norman Jones, Division of Pure Chemistry, National Research Council, Ottawa. Due to the smallness of the samples, the only definite finding was the absence of a Δ^4-3 keto group absorption in both samples. In addition both showed a broad irregular band between 1710 and 1730 cm^{-1}. The rest of the spectrum was not detailed enough for us to make any comments.

From the evidence presented so far, the following tentative conclusions could be drawn regarding the chemical structure of metabolites A_1 and A_2:

a. *Side chain of* A_1 *and* A_2.—The side chain of both compounds reduces blue tetrazolium in the cold, which indicates an α-ketol group. CrO_3 oxidation fails to produce a carbonyl compound. This excludes the presence of a hydroxyl group at C_{17}. The side chain has an easily acetylable group which, however, is labile to cold alkali as demonstrated by the reducing properties of the acetates. HIO_4 oxidation does not produce a carbonyl compound. The only known side chain meeting all these requirements is a —CO—CH_2OH group. However, as both oxidants produce products insoluble in saturated bicarbonate, the presence of an oxygen function at C_{18} must be postulated. This function might mask a —COOH group formed by oxidation through the formation of a lactone.

b. *Ring A of* A_1 *and* A_2.—Neither metabolite absorbs ultraviolet light. The infrared, concentrated sulphuric and 100% phosphoric acid spectra fail to show absorptions characteristic for the Δ^4-3 keto group. CrO_3 produces different products than does HIO_4, which argues in favor of presence of a hydroxyl group

* Mobility relative to the solvent front.

affected by the former but not by the latter oxidant. This hydroxyl might very well be situated at C_3 and would account for the blue Zimmermann reaction given by the CrO_3 oxidation products. A C_3—OH would also be less susceptible to acetylation than a C_{21}—OH; hence the two different acetates produced by ordinary and by the Mattox acetylation. All this excludes the presence of a cross-conjugated system but is very well in agreement with a C_3—OH group attached to a saturated ring system. No differences could be found between metabolite A_1 and metabolite A_2 except in chromatographic mobilities. Theoretically the α,β-unsaturated carbonyl group in ring A can be reduced to saturated alcohols which may occur in all of the four possible stereochemical configurations that exist at the asymmetric centers at C_3 and C_5. Dorfman, and Dorfman and Ungar (19, 20), pointed out that C_{21},17-desoxy steroids when containing a cross conjugated system in ring A are reduced biologically to a mixture of pregnane (5β) and allopregnane (5α) derivatives. On the other hand,

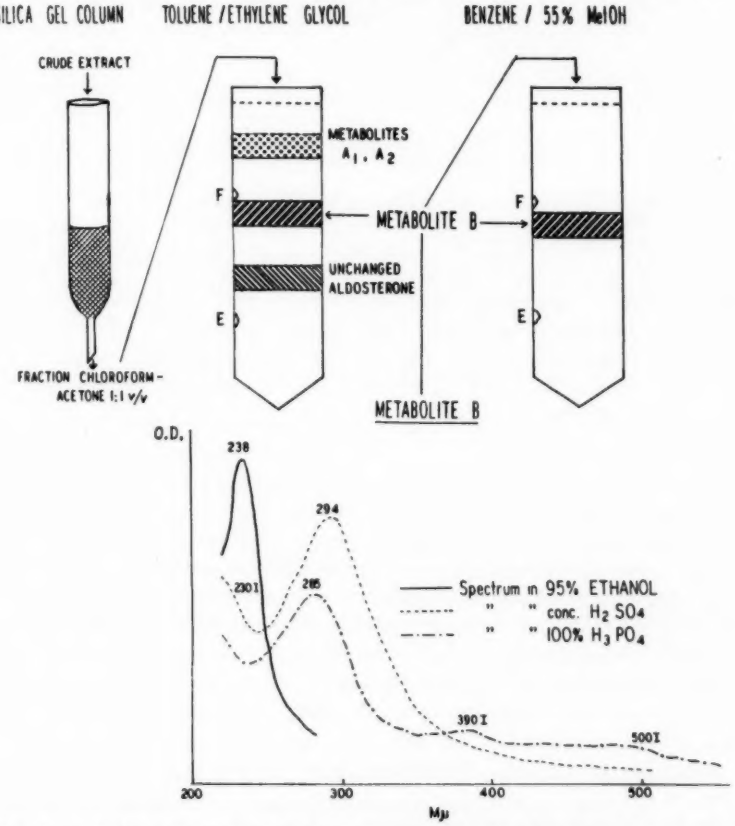

FIG. 2. Isolation of metabolite B following the incubation of *d*-aldosterone with dog liver slices. Absorption curves of metabolite B in 95% ethanol, concentrated sulphuric acid, and 100% phosphoric acid are shown.

cortisol perfusion through dog liver has shown a variety of pregnane products (6), contrary to the findings with rat liver perfusions (4, 14, 15, 16, 17) where the allopregnane series seemed to predominate. It is entirely logical to suppose that A_1 and A_2 are stereoisomers. This problem will be discussed again in connection with the human liver slice experiments (vide infra).

c. Configuration at C_{11} *and* C_{18}.—There is no direct evidence at hand for the configuration of these two carbon atoms in A_1 and A_2. However, as already mentioned, oxidants did not produce acidic compounds from either metabolite. If the side chain is really the proposed one, this must indicate the possibility of lactone formation between C_{18} and the side chain.

B. Metabolite B

The isolation of this metabolite is shown in Fig. 2. On the chromatograms, metabolite B was localized by scanning with ultraviolet light.

Only about 25 μg of this substance were isolated, and the number of characterization procedures was necessarily limited.

1. Ultraviolet absorption: λ_{max}^{EtOH}: 238 mμ.
2. Blue tetrazolium reduction: negative.
3. Spectrum in concentrated sulphuric acid: maxima: 230 I, 294; minimum: 245 mμ.
4. Spectrum in 100% phosphoric acid: maxima: 285, 390 I, 500 I mμ; minimum: 235 mμ.
5. Paper chromatographic mobilities:
   toluene/ethylene glycol: R_{cort}: 0.48,
   benzene/55% methanol: R_f : 0.26.

The ultraviolet absorption together with the peak absorptions in concentrated acids suggests strongly a Δ^4-3 keto grouping in ring A. There is no evidence of an α-ketol side chain. The relative polarity of the compound places it rather in the C_{21} than the C_{19} series. This high polarity could also indicate a glycol type side chain.

C. Free Aldosterone

The appropriate zone was eluted from the toluene–ethylene glycol chromatograms and successively chromatographed in the *t*-butanol–isooctane/water and benzene/55% methanol paper systems. It was finally purified on a Kieselgur column. In all paper systems, it had identical mobility with authentic free aldosterone and it was homogeneous in all these systems.

The isolated substance showed the following physicochemical characteristics:

1. Ultraviolet absorption: λ_{max}^{EtOH}: 239 mμ.
2. Blue tetrazolium reduction: positive.
3. Spectrum in concentrated sulphuric acid: maximum: 288 mμ; minimum: 240 mμ.
4. Spectrum in "100%" phosphoric acid: maximum: 280 mμ; minimum: 245 mμ.
5. Paper chromatographic mobilities:
   toluene/ethylene glycol: $R_{hydrocort}$: 2.00,*

* Mobility relative to hydrocortisone.

t-butanol–isooctane/water: R_{cort} : 0.66,
benzene/55% methanol: R_f : 0.39.

6. Acetylation: The substance was acetylated according to the procedure of Mattox *et al.* (33). The monoacetate still reduced blue tetrazolium and had identical mobility with authentic *d,l*-aldosterone-21-monoacetate on the cyclohexane-benzene/propylene glycol paper system.

From the evidence presented above it was concluded that this material was indeed identical with free aldosterone. No aldosterone monoacetate was recovered even in the cases when this compound was incubated.

Dog Liver Slices Incubated with Aldosterone-21-C^{14}

From the foregoing experiments it could be concluded that "in vitro" *d,l*- and *d*-aldosterone and *d,l*-aldosterone-21-monoacetate is metabolized to two major reduced metabolites, A_1 and A_2, by surviving dog liver tissue. Though none of the metabolites was found in control experiments—either involving tissue without substrate or inactivated tissue with the steroid—it was decided to secure more direct evidence that these transformation products originate indeed from aldosterone by metabolic transformation through liver enzyme systems.

A preliminary, qualitative experiment was performed incubating dog liver slices with aldosterone-21-C^{14}.*

Nine micrograms of labeled aldosterone (600 c.p.m./μg) were incubated with 318 mg of dog liver slices for 4 hours using the usual technique.

After incubation the mixture was extracted as usual and the crude chloroform extract chromatographed for 60 hours on the toluene–ethylene glycol system.

The chromatographic paper was scanned for radioactivity and an activity concentration was found at a position of R_{cort}: 0.21. At the place where unchanged aldosterone should have migrated ($R_{hydrocort}$: 2.00) only traces of activity were present.

The R_{cort}: 0.21 spot was eluted from paper and rechromatographed in the benzene/55% methanol system. Here an active spot was detected at R_{cort}: 0.39. Only traces of activity were present in more polar positions. No activity was detectable in the overflow of the toluene – ethylene glycol chromatogram.

The above-mentioned positions very closely agree with the positions occupied by metabolite A_2. This and the almost complete disappearance of the C^{14} aldosterone seemed to prove that A_2 is indeed a true metabolite of aldosterone. Parallel experiments with liver slices from the same donor dog with *d,l*-aldosterone–monoacetate yielded A_1 and A_2 as usual. We could not find any explanation for the absence of A_1; unless it was the very high tissue to substrate ratio (35,000:1).

Human Liver Slices Incubated with Aldosterone

Human liver slices were incubated with *d,l*-aldosterone-21-monoacetate and *d*-aldosterone isolated from human late pregnancy urine.

* This experiment was made possible through the kind co-operation of Drs. C. J. P. Giroud and J. L. M. Stachenko of the Endocrine Research Laboratory, Montreal Children's Hospital, Montreal, Que., who made the labeled aldosterone available and performed the scanning of the chromatograms.

Two incubations using the synthetic monoacetate were performed, using 530 and 750 μg of substrate with a tissue to substrate ratio of 1500:1. *d*-Aldosterone was incubated with human liver slices in two experiments, with the following amounts of substrate and substrate to tissue ratio: (1) substrate: 740 μg substrate to tissue ratio 1:2000; (2) substrate: 1000 μg substrate to tissue ratio 1:4600. In the course of these experiments, three metabolites were isolated in addition to free, intact aldosterone. One of these metabolites proved to be identical with metabolite A_2 isolated from dog liver experiments. The second metabolite, though having the paper chromatographic polarity of metabolite A_1 was isolated in such small quantities that its identity with A_1 of canine origin could not be established.

The quantitative yield of the metabolites is shown in Table III. It can be seen that, contrary to the dog liver experiments, A_1 and A_2 are not formed in

TABLE III

Different forms of aldosterone incubated with human liver slices. Quantitative yield of metabolites

Substrate	Metabolites, yield in %			Recovery of free aldosterone, %
	A_1	A_2	C	
d,l-Aldosterone-21-monoacetate	Traces	3.2–4.7	4.4	Traces
d-Aldosterone	2.5	10	—	Traces

roughly equal quantities; in human liver incubations A_2 seemed to be the predominant transformation product. No metabolite C was found in incubations using *d*-aldosterone.

Isolation and Partial Chemical Characterization of Metabolites

A. Metabolites A_1 and A_2

It has to be noted that only semiquantitative determination of the substances isolated were performed by visual estimation of the color intensities of spots on paper following spraying with blue tetrazolium. This method has an inherent error of at least ± 25%. The incubations, extraction, and isolation procedures were performed as described earlier for the dog experiments.

The identity of human metabolite A_2 with that of canine origin was established by the following criteria: (1) chromatographic mobilities in two systems; (2) blue tetrazolium reduction; (3) spectrum in concentrated sulphuric acid; (4) acetylation (acetic anhydride in pyridine for 48 hours at room temperature) and chromatographic mobility of acetate; (5) mixed chromatograms of the substance and its acetate with A_2 and A_2 acetate from dog experiments.

A compound having the mobility of metabolite A_1 was present in all human experiments in rather small quantities only (from traces to 25 μg incubation). Consequently, on this substance, only chromatographic mobility studies could be performed; its identity with A_1, though probable, could not be established.

B. Metabolite C

The isolation of metabolite C from incubations of *d,l*-aldosterone-21-monoacetate with human liver slices is shown in Fig. 3. The metabolite was located

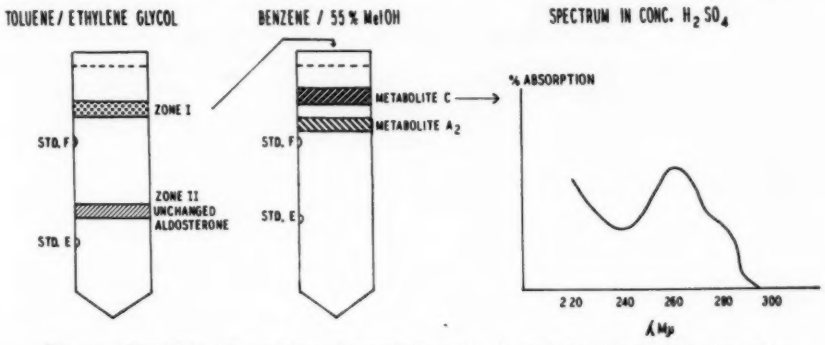

FIG. 3. Zone I is the position of metabolites A_1, A_2, and C. Zone II is that of unchanged free aldosterone. In cases when metabolite C was detected, A_1 was present only in trace amounts (see Table III).

by ultraviolet light scanning. Only about 25 μg were isolated, consequently the chemical characterization of this substance could only be done in a very limited way. Metabolite C showed the following physicochemical characteristics:

1. Ultraviolet absorption: positive on paper.
2. Blue tetrazolium reduction: negative on paper.
3. Chromatographic mobilities:
   toluene/ethylene glycol: R_{cort}: 0.20,
   benzene/55% methanol: R_{cort}: 0.20.
4. Acetylation (acetic anhydride in pyridine, at room temperature for 48 hours): acetate formed.
   Mobility of acetate in the benzene/55% methanol system: R_{cort}: 0.43.
5. Spectrum in concentrated sulphuric acid: maxima: 265, 2751; minimum: 240 mμ.

No conclusions can be drawn as to the configuration of this substance until more material will be available.

ISOLATION OF ALDOSTERONE METABOLITE FROM HUMAN URINE

The foregoing experiments established the presence of at least one reduced aldosterone metabolite (A_2) in incubation mixtures using human liver slices and free and acetylated aldosterone. This and the work of Ulick and Lieberman (54, 55) prompted us to start a search for a ring A reduced, α-ketolic aldosterone metabolite in human urine.

Method (see also Fig. 4): The urine of a patient suffering from primary aldosteronism was selected for this study (23, 27*a*). The aldosterone excretion of this patient was rather high, varying between 30 and 120 μg/24 hours. A 48-hour urine sample was hydrolyzed first with animal β-glucuronidase

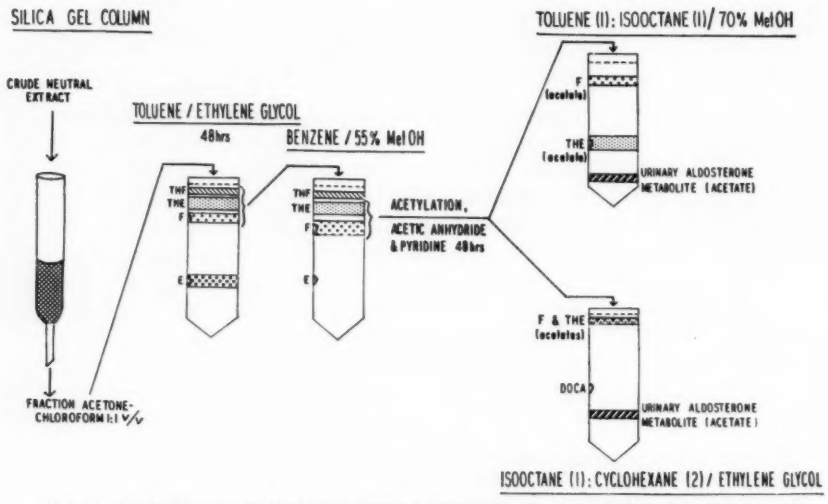

FIG. 4. Isolation of a reduced aldosterone metabolite from the urine of a patient with primary aldosteronism. The metabolite was isolated in acetate form.

(300 Fishman units/ml) for 24 hours at 37° C followed by continuous extraction at pH 1 for 30 hours. The chloroformic extracts were pooled, washed with 0.1 *N* NaOH and water, and evaporated under reduced pressure at 40° C.

The crude neutral extract was first chromatographed on the silica gel column in the same manner as described previously for the incubation extracts. The chloroform:acetone 1:1 eluent was first chromatographed on a toluene – ethylene glycol paper system for 48 hours.

It has been established previously that the position of A_1–A_2 in this system coincides very closely with that of tetrahydrocortisone. Consequently, the whole upper portion of the chromatogram from the starting line to the lower limit of the hydrocortisone zone was eluted and rechromatographed in the benzene/55% methanol system.

On this strip the following major α-ketolic urinary compounds were now present:

tetrahydrohydrocortisone: R_{cort}: 0.20,
tetrahydrocortisone : R_{cort}: 0.36,
hydrocortisone : R_{cort}: 0.52,

metabolite A_1 ? : R_{cort}: 0.20,
metabolite A_2 ? : R_{cort}: 0.43.

Again the upper portion of the chromatogram from the starting line to the lower limit of the hydrocortisone zone was eluted.

The eluate was acetylated with acetic anhydride in pyridine for 48 hours at room temperature. The acetylated mixture, after preliminary washing, was chromatographed in the toluene–isooctane/70% methanol system. Here the acetylated aldosterone metabolites separate easily from tetrahydrocortisone–diacetate (R_{THEAc}: 1.17).

However, later on a more suitable paper chromatographic system was set up for the separation of the acetylated metabolites from THEAc. It must be kept in mind that the problem is complicated by the fact that tetrahydrocortisone is present in urines in the mg range, while the aldosterone metabolite only in the μg range. The new paper chromatographic system consisted of ethylene glycol as stationary phase and a 1:2 v/v mixture of cyclohexane–isooctane as mobile phase. The chromatographic paper was impregnated with a 1:1 mixture of ethylene glycol and methanol and the chromatogram run for 5 hours. In this system, tetrahydrohydrocortisone–diacetate, tetrahydrocortisone–diacetate did not migrate at all. Desoxycorticosterone acetate migrated 24 cm, while A_1 and A_2 acetate migrated 34 cm in 5 hours (R_{DOCA} of acetylated metabolites: 1.41).

By the above described means, 70 μg of an acetylated compound having the mobility of metabolite A_1–A_2 acetates was isolated. The compound was further purified by high vacuum distillation. This compound reduced blue tetrazolium did not absorb ultraviolet light, and in concentrated sulphuric acid gave the following spectrum: maxima: 265 I, 315, 410; minima: 240, 370 mμ.

The presence of this material could be demonstrated in the urine of normal subjects and hypertensive patients, though only in minimal amounts (less than 10 μg/24 hour).

Discussion

It has been established by several groups that the principal metabolic activity involving Δ^4-3 keto, α-ketolic C_{21} steroids of adrenocortical origin is the reduction of the ring A cross conjugated system to C3-hydroxyl groups in mammals. This transformation has been shown to be true in vivo in humans (8, 22, 24, 53) and in vitro involving liver tissue preparations or liver perfusions in several species (21). It appears that the above reaction is necessary for the conjugation and subsequent renal elimination of these metabolites from the body. The main site of activity has been definitely established to be in the liver (16, 17, 48, 49, 52). It was a logical supposition that, at least partly, aldosterone is metabolized by the same mechanism.

The evidence presented in this study showed that in "in vitro" systems involving liver slices of canine and human origin aldosterone is partly transformed to ring A reduced, α-ketolic substances. This transformation will entail loss of biological activity. This has been demonstrated on aldosterone by the experiments of Chart *et al.* (18) cited earlier.

There is no direct evidence concerning the steric orientation of the C3—OH,5C system in the metabolites herein described. Axelrod (6) has shown that dog liver tissue favors the 3α-pregnane series and the metabolic products of cortisone and cortisol isolated from human urine are preponderantly of the pregnane series. However, in the last 2 years several groups have demonstrated that 3α-allo-tetrahydro hydrocortisone is present in and forms a regular part of the urinary steroid metabolite pattern in humans (12, 43, 44). The very similar physicochemical characteristics of metabolites A_1 and A_2 suggest that they are stereoisomers. The assignment of the allopregnane configuration to A_1 and the pregnane configuration to A_2 would very well agree

with the relative scarcity of the former and preponderance of the latter in human liver slice experiments.

The identity of A_2 with the aldosterone metabolite pregnane-3α, 18,21-triol,-11,20-dione (54) has not been fully established. The chromatographic mobility data are very similar and there is a similarity in the very large change in polarity after acetylation shown by both compounds, pointing toward a possible triacetate formation.

Metabolites B and C, isolated in trace amounts, seem to be 20-glycol derivatives of aldosterone. It must be kept in mind that the metabolism of aldosterone most probably gives rise to a whole new family of C18 oxygenated steroids with unknown physicochemical characteristics. In addition, the possibility of the metabolic conversion of aldosterone to 11-dehydroaldosterone (18-oxo-Δ^4-pregnane,21-ol,3,11-dione; isolated from human urine by Nowaczynski *et al.* (36)) cannot be excluded. In this case 11-dehydroaldosterone might produce a separate family of metabolites again with C18 oxygen function.

The importance of the investigation of the metabolism of this hormone lies in the fact that (1) as it has been clearly demonstrated by Ulick and Lieberman (55) the assay of urinary aldosterone alone does not reflect its production rate by the adrenals, and (2) as pointed out by Yates *et al.* (58) and Forchielli *et al.* (26), the rate of adrenal cortical secretion may be very largely determined in many instances by the capacity of the liver to inactivate the Δ^4-3 keto steroids by ring A reduction.

Conclusion

The metabolic activity of dog and human liver slices have been investigated on *d,l*-aldosterone-21-monoacetate, *d,l*-aldosterone, *d*-aldosterone, and *d*-aldosterone-21-C^{14} in a Krebs–Ringer–phosphate–glucose (200 mg%) medium. Dog liver slices partially metabolized either form of aldosterone under these conditions to two ring A reduced, α-ketolic substances, designated as metabolite A_1 and metabolite A_2.

Human liver slices partly transformed *d,l*-aldosterone-21-monoacetate and *d*-aldosterone to metabolite A_2, while metabolite A_1 was only present in traces.

Preliminary experiments using C^{14} labeled, natural free aldosterone with dog liver slices clearly indicated that at least A_2 is a true metabolite of aldosterone.

Two additional, non α-ketolic, ultraviolet light absorbing transformation products were isolated, one originating from a dog liver slices – *d*-aldosterone (metabolite B), the other from a human liver slices – *d,l*-aldosterone-21-monoacetate system (metabolite C).

The partial physicochemical characterization of the four metabolites was attempted. The urine of a patient suffering from primary aldosteronism yielded a substance probably identical with A_2.

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EFFECT OF LIPOTROPIC AGENTS UPON THE NET SYNTHESIS OF CHOLESTEROL IN THE RAT[1]

SAILEN MOOKERJEA[2] AND C. C. LUCAS

Abstract

The content of cholesterol in the bodies of young male rats fed a basal hypolipotropic diet has been compared with that of other rats pair-fed on similar diets supplemented with choline chloride, methionine, or vitamin B_{12}, respectively. Increased efficiency of utilization of the food and variable lipotropic effects were observed, but no significant difference was found in the total amounts of cholesterol at the end of the 3-week test periods. The lipotropic agents do not influence to any significant degree the net biosynthesis of cholesterol in rats fed this type of diet but they do affect the distribution, tending to keep the liver lipids, including cholesterol, in the normal range.

Introduction

The effect of a deficiency of the lipotropic factors upon the deposition of abnormal amounts of fat in the liver is well known (1, 2, 3, 4, 5, 6, 7). Choline is more effective in preventing an increase in hepatic glycerides than in cholesteryl esters, especially if the diet contains appreciable amounts of cholesterol. The concentration of cholesterol in the serum decreases in animals maintained for long periods on hypolipotropic diets; the presence of choline in the diet prevents this fall (8). Although it has long been known that lipotropic agents are concerned in the metabolism of glycerides and cholesteryl esters, the evidence, particularly with reference to cholesterol, has never permitted an unambiguous answer as to whether the effect is primarily on biosynthesis, utilization, or transport.

Guggenheim and Olson (9) gave C^{14}-labelled acetate to rats fed diets containing or lacking choline. Their data do not show any specific effect of choline upon the incorporation of isotopic carbon of acetate into the cholesterol of the liver and suggest that the ability to metabolize newly synthesized fat decreases as the duration of choline deficiency is prolonged.

In the present study the effects of choline, methionine, and vitamin B_{12} upon the net synthesis and distribution of cholesterol in young male rats fed purified diets have been examined. Supplementation of a basal hypolipotropic diet with these agents, one at a time, failed to change significantly the amount of cholesterol found in the rats after a 3-week period, although the distribution of cholesterol in the body was affected.

Methods

Dietary Conditions

Male rats (Wistar strain, initial weights from 74 to 124 g) were kept in individual cages and given fresh food and water daily. Leftover food and scatter were weighed each day and the food intake of each rat was estimated.

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Paired-feeding was done on a group basis, i.e., the average amount consumed by the rats fed the deficient diet was offered to those given the corresponding supplemented diets. Further details of the feeding and the care of the animals have been reported elsewhere (8, 10, 11).

The percentage composition of the basal diet was alcohol-extracted peanut meal (solvent process) 12, soya protein (Drackett) 8, casein (vitamin-free) 1, L-cystine 0.2, salts 3, celluflour 1, corn starch 10, dextrin 10, sucrose–vitamin mixture 1, sucrose 38.8, hydrogenated fat (Primex) 10, corn oil 5, vitamins A, D, and E in corn oil; (for details see Refs. 11, 12). The ration supplied 15% of protein and the methionine content (190 mg per 100 g) appeared to be adequate for optimal growth of the rats when choline was present (11). The vitamin mixture used in the present study differed from that used by Young *et al.* (11) in that vitamin B_{12} was omitted. When supplements of choline or methionine were added to the diets (Table I) an equal weight of sucrose was omitted. In experiment KB supplementary vitamin B_{12} was added to the sucrose–vitamin mixture. The casein used in that experiment was treated with hot acetic acid and with different dilutions of alcohol (75, 80, 90, and 100%) to eliminate as far as possible any vitamin B_{12} or B_{12}-like factors.

Extraction and Analysis of Lipids

Blood (3 to 8 ml) was obtained at the time of killing from the carotid artery of fasting anaesthetized rats; the clot (separated from the serum) was weighed and added to the carcass. Cholesterol was determined in the lipids extracted from the serum with Bloor's alcohol–ether mixture (8). The liver was excised, wiped free from blood, blotted, weighed, and analyzed separately.

The tail and skin were cut into small pieces which, with the carcass, were put through a Hobart meat grinder several times, using the cutting plate with large holes twice and the medium cutting plate once. All equipment used in the grinding was washed with acetone to recover adherent lipid. In one experiment the kidneys were analyzed separately.

The extraction and analysis of the lipids from the liver and other organs have been described more fully elsewhere (8, 10). In brief, preliminary treatment with acetone (5–7 ml/g) was followed by at least three extractions with hot ethanol (5 ml/g for at least 5 minutes each time). The combined extracts were taken to dryness *in vacuo* (water bath 45°) and the crude lipid residue was treated several times with small portions of a 3:1 (v/v) mixture of petroleum ether (b.p. 40–60°) and chloroform. The resulting solution of rectified lipids was centrifuged. The clear supernatant was decanted and made up to a convenient volume with the same solvent mixture (50 ml for livers, 250 ml for carcasses).

Results

Table I shows the dietary supplements and includes data on food intake, gains in weight, and survival. The concentration of free and total cholesterol (mg/100 ml) found in the serum of the rats on the different experimental regimens is given in Table II. In the absence of choline, the concentration of total cholesterol in the serum fell, confirming earlier findings (8). Choline in the diet

TABLE I

Experimental conditions
(Male rats were fed the test diets for 21 days; experiment KB ran for 28 days; all rats were fasted for 16 to 20 hours before killing)

Expt.	Group	Supplements: Choline chloride (% in diet)	Supplements: Methionine (% in diet)	Supplements: Vitamin B_{12} (μg/100 g)	Surviving rats /starters	Body weight (g): Initial	Body weight (g): Gain	Food intake (g/rat/day)
K	0	—	—	—	9/10	117	56	11.6
	1*	0.36	—	—	10/10	119	67	11.4
KA	2	0.04	—	—	8/10	92	62	11.3
	3*	0.04	0.06	—	5/5	83	74	11.0
	4*	0.04	0.24	—	10/10	84	81	11.1
	5*	0.04	0.48	—	5/5	81	87	11.4
	6	0.04	0.24	—	5/5	76	101	13.6
KB	7	0.04	—	—	8/10	92	79	11.9
	8*	0.04	—	0.75	9/10	88	88	10.9
	9*	0.04	—	15	10/10	86	89	11.0
	10	0.04	—	15	3/4	84	87	11.8

NOTE: 10 normal rats (98 to 128 g, mean 114 g) raised on commercial ration were killed as controls for expt. K; 5 normal rats (70 to 84 g, mean 76) controls for KA and KB.
* Pair-fed with others in same experiment.

TABLE II

Fasting blood cholesterol (mg/100 ml serum)

	Free cholesterol	Total cholesterol*
Controls for K (10 normal rats fed chow)	15	76 ± 7.4
K0	11	47 ± 11.3
K1	10	74 ± 18.6
Controls for KA and KB (5 normal rats fed chow)	11	65 ± 2.3
KA2	10	46 ± 7.3
KA3	8	45 ± 10.6
KA4	10	64 ± 9.8
KA5	13	71 ± 19.2
KA6	11	68 ± 15.7
KB7	10	52 ± 8.6
KB8	8	49 ± 7.6
KB9	10	54 ± 8.6
KB10	13	65 ± 10.6

* Mean value ± standard deviation.

maintained the value and methionine had a similar effect when adequate dosage was used. Results in the case of vitamin B_{12} are less clear cut except for those fed ad libitum (group 10, 15 μg vitamin B_{12}/100 g diet).

The data in the upper part of Table III (experiment K) reveal no significant effect of choline on the accumulation of newly synthesized cholesterol in the body. It has long been known that in the absence of choline from the diet more cholesterol is found in the liver than in its presence. The data in Table III show that a corresponding amount of cholesterol disappears from the carcass. However, these data do not permit us to decide whether dietary choline (*a*) *improves* (1) transport of cholesterol from the liver, or (2) utilization in the liver, or whether (*b*) it *interferes* (1) with transport to the liver or (2) with hepatic synthesis.

TABLE III

Absolute amounts of total lipids and cholesterol at end of experiments

	Total lipids (g)				Total cholesterol (mg)			
	Carcass	Liver	Kidney	Total*	Carcass	Liver	Kidney	Total*
K0	17.13	4.720	0.103	21.96	341	44	8	394 ± 33.9
K1	22.89	0.396	0.084	23.38	364	18	7	390 ± 25.4
Chow†	8.90	0.288	0.068	9.27	254	11	5	271 ± 26.5†
KA2	16.04	4.41	—	20.46	319	43	—	363 ± 27.3
KA3	17.40	1.87	—	19.28	316	21	—	338 ± 28.7
KA4	18.40	0.88	—	19.29	329	23	—	353 ± 31.1
KA5	19.20	0.41	—	19.62	345	16	—	362 ± 29.4
KA6	23.20	0.64	—	23.85	373	23	—	397 ± 24.1
KB7	17.71	4.09	—	21.81	343	45	—	389 ± 44.4
KB8	22.17	3.23	—	25.41	320	39	—	360 ± 19.5
KB9	21.02	2.00	—	23.12	311	34	—	346 ± 21.2
KB10	21.11	0.98	—	22.20	340	26	—	367 ± 36.3
Chow‡	3.28	0.18	—	3.47	192	9	—	202 ± 13.0‡

*The total amount of lipid and of cholesterol in the small portion of blood taken at killing was calculated (7–11 mg and 0.6–1.4 mg, respectively) and is included in the total values shown. The clot was weighed and returned to the carcass before the latter was extracted. Significance of differences (cholesterol):

KA3 vs. KA2 : $P = 0.15$,
KA6 vs. KA2 : $P = 0.1$,
KB9 vs. KB7 : $P = 0.02$.

†These control rats weighed on the average only 114 g while those of group K0 weighed 173 g and those of group K1 weighed 186 g when killed.

‡These control rats when killed weighed only 76 g, on the average, i.e. scarcely one-half that of the final weight of the rats of experiments KA and KB.

Supplements of methionine had a definite effect in improving the efficiency of utilization of the food consumed (Table I, experiment KA), but had no clear-cut effect upon the total cholesterol. Methionine, like choline, tended to keep the concentration of cholesteryl esters in the liver low, with correspondingly more being found in the carcass.

Vitamin B_{12} had no stimulatory effect upon the net synthesis of cholesterol by the rat (Table III, experiment KB), although like methionine, it improved the efficiency of utilization of the food consumed. Slightly less cholesterol was found in the livers of the rats given vitamin B_{12} than in those of the controls and the decrease in the total body is considerably greater.

Discussion

The data fail to provide any evidence that dietary choline, methionine, or vitamin B_{12} exert a determining influence upon the net biosynthesis of cholesterol in the intact rat. The abnormal accumulation of cholesteryl esters present in the liver of the choline deficient rat occurs apparently at the expense of cholesterol that would normally be found elsewhere in the body. Including choline in the ration caused a redistribution but no change in the total amount of cholesterol found in these rats.

Acknowledgments

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ACETYLASPARTIC ACID AND AMMONIA POISONING

JEAN-PAUL DU RUISSEAU

Abstract

Acetylaspartic acid has been followed in nine tissues of normal and ammonia-poisoned rats. This acetylated amino acid is present at high concentrations in normal brain and remains unchanged in ammonia-poisoned brain. Acetylaspartic acid is absent or present in trace amounts in normal liver. It increases appreciably in ammonium acetate poisoning, reaching a peak at death. No change in concentration was detectable in the other tissues examined. There is a correlation between aspartic and acetylaspartic acid in the liver. But no correlation was observed between acetylaspartic acid on the one hand and ammonia and urea on the other. The possible origins of acetylaspartic acid are discussed.

Introduction

During the course of studies on ammonia poisoning in rats, it was deemed interesting to follow the organic acid distribution in various tissues using paper chromatographic techniques. It happened that the normal brain chromatograms contained a spot that could not be readily identified. This substance was also present in traces in normal liver and kidney. If, however, the rat was injected with an $LD_{99.9}$ dose of ammonium acetate, this unknown substance did not vary in the brain, but increased appreciably in the liver. This suggested that this unidentified substance was involved in ammonia poisoning. The isolation and subsequent identification of this metabolite form the basis of this communication.

Materials and Methods

Animals

Male Sprague–Dawley rats weighing approximately 200 g were used throughout this study. Previous to use, they were always starved for 24 hours, but given water ad libitum. The injection of the various agents tested, the sacrifice of the animals, the collection of tissues, and their preparation as alcoholic homogenates were carried out as described in previous studies (1, 2, 3) and therefore will be omitted here.

Chromatography

In general, the method used for the chromatography of the alcoholic extracts of the rat tissues is the one described by Nordmann *et al.* (4). First, the unknown homogenate is passed on a small column of Dowex 2 whereby neutral and alkaline substances are eliminated and then the acidic compounds are eluted with 12 *N* formic acid. The eluate is evaporated to remove the formic acid and the dry extract is redissolved in a small volume of water; the resulting solution is spotted on paper, using an automatic sample depositor (5).

Solvents: 1.	Absolute ethanol	80 vol.
	Ammonia 21.6%	5 vol.
	Water	15 vol.

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Contribution of the Laboratoire de Biochimie, Institut de Gérontologie, Hôpital Notre-Dame de la Merci, 555 Gouin Blvd. West, Montreal, Que.

2. *n*-Propanol	50 vol.
Eucalyptol	50 vol.
Formic acid 98%	20 vol.
Water (until solvent becomes turbid)	

The two solvent systems are used consecutively in a two-dimensional paper chromatogram. After drying, the chromatograms are dipped into 0.1% solution of bromocresol green in acetone. The acids appear as a yellow spot on a green background and were estimated as follows: six bidimensional chromatograms were made: (*a*) The equivalent of 0.7 g of homogenate was deposited on two of them, after having passed through Dowex 2, etc. as described earlier in the method. (*b*) Two other chromatograms were made as in "*a*" above, but 100 micrograms of a known pure acetylaspartic acid had been added to the homogenate. (*c*) Finally, two more chromatograms were run with an eluate which corresponded to 100 micrograms of acetylaspartic acid.

All these chromatograms were run simultaneously in identical conditions; they were then dried and stained as described earlier in the method. The spot corresponding to acetylaspartic acid was eluted with 5 cc of 20% methanol. Each solution was then titrated by the method of Kolthoff (6) with 0.001 *N* NaOH, using a Gilmont ultra-microburet and a Beckman Model G potentiometer, the titration always being carried out under a stream of nitrogen.

Determination of NH_3 *and Urea* N

Ammonia and urea N were determined by the Conway procedure (7).

Aspartic Acid

Aspartic acid was determined by a paper chromatography method described in a previous study (3).

Experimental

Six animals were injected with an $LD_{99.9}$ dose of ammonium acetate (830 mg per kg of body weight) and sacrificed at 5-minute intervals until death (which generally occurred 15 minutes after the injection of ammonium acetate). Another group of 12 rats were injected with 4 mmoles/kg of L-arginine.HCl and 1 hour later with an $LD_{99.9}$ dose of ammonium acetate. In these conditions, 100% survived. Again they were sacrificed at various time intervals. Other groups of animals were set up similarly; however, 1 hour prior to receiving an $LD_{99.9}$ dose of ammonium acetate, they were injected with either 1 mmole/kg of L-arginine.HCl (in which case most of them died) or 1 mmole/kg of L-arginine.HCl plus 4 mmoles/kg of NaCl, where 65% survived.

Paper chromatograms of organic acids of normal and ammonia-poisoned rat tissues were compared. They usually showed succinic, fumaric, malic lactic, pyrrolidone carboxylic, and glutamic acids. However, in addition to the latter, the normal brain contained an acidic substance which was very close to the malic acid spot and was present at relatively high levels (see Fig. 1). This unknown, which normally was almost absent from the normal liver, increased very appreciably in the liver of ammonia-poisoned animals, whereas it did not seem to vary in the brain under the same conditions. Therefore, as this unknown

seemed to be correlated with toxic levels of ammonia in the liver, attempts were made to isolate and identify it.

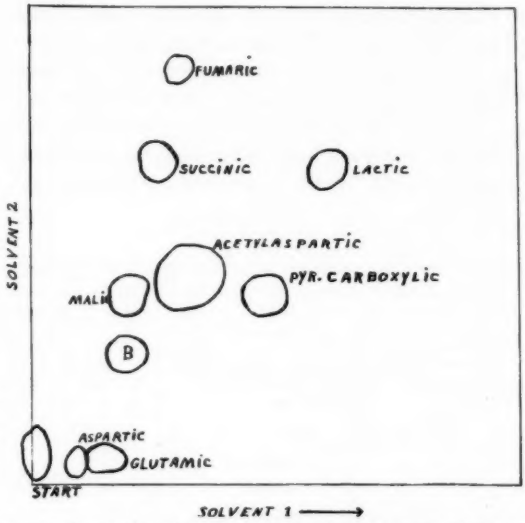

FIG. 1. Diagram of organic acids obtained by depositing an eluate corresponding to 0.7 g of brain tissue. Spot B is an acid that could not be identified. Solvent 1 is alkaline and solvent 2 is acidic. For details see text.

Isolation and Identification

Fifty milliliters of the normal brain homogenate (5 g of brain) were passed on a Dowex 2 column in the formate form and subsequently eluted with 300 ml of 3 *N* formic acid at a rate of 0.5 ml/minute. The fractions were collected over a Technicon fraction collector (7 ml per tube). All tubes were evaporated to dryness in vacuum and dry extracts redissolved in 1 ml of water. The solution in each tube was chromatographed on bidimensional chromatograms using our usual solvents. The fifth tube consisted exclusively of the unknown. Consequently, the latter was dried in vacuum and the dry extract (1.5 mg) used for analysis.

Identification

1. The fact that it was adsorbed on Dowex 2 (anionic resin) and that it was stained yellow by bromocresol green indicated that the compound was acidic.
2. The nitrogen content was 8%.
3. It was ninhydrin negative when run on paper chromatograms, but after hydrolysis the bromocresol positive spot disappeared and a ninhydrin-positive spot appeared. This spot was eluted and rechromatographed against aspartic acid in two different solvents. Both migrated identically and both had the characteristic ninhydrin color of aspartic acid.
4. The titration curve yielded only one point of inflection and showed the normal brain content to be 100 mg/100 g (expressed as acetylaspartic acid).

5. The spots of the normal brain unknown and the ammonia-poisoned rat liver unknown which were suspected to be the same were cut from the paper chromatograms, eluted, and run simultaneously in the bidimensional chromatographic system. They were shown to have the same migrational characteristics.

A publication by Tallan *et al.* (8) reported the presence in the cat and the rat brain of N-acetyl-L-aspartic acid at concentrations of about 100 mg/100 g and mentioned that this compound was almost absent (1–3 mg/100 g) in liver, kidney, muscle, or urine. The similarity to our unknown was obvious and consequently we made various comparisons between our unknown and a known synthetic acetylaspartic acid. Table I summarizes the results.

TABLE I

Comparison between the unknown in normal brain and acetylaspartic acid

	Unknown	Known N-acetyl-DL-aspartic acid
Occurrence and concentration	In rat brain 100 mg/100 g almost absent from normal liver	Reported by Tallan *et al.* to be present in normal brain at about 82 mg/100 g and 1–3 mg in the normal liver
Nitrogen content	8%	8%
Bromocresol green	Positive	Positive
Ninhydrin	Negative	Negative
Hydrolysis in 5*N* HCl	Acidic spot disappeared and aspartic acid appeared	Acidic spot disappears and aspartic acid appears
Chromatographic behavior:		
R_f in solvent 1	0.35	0.35
R_f in solvent 2	0.39	0.39

Results and Discussion

The comparison of the occurrence and properties of the unknown acidic substance with acetylaspartic acid shows that this unknown acid is acetylaspartic acid. This fact is interesting because this metabolite, which is almost absent from normal liver, appears at appreciable concentrations in the liver of rats poisoned with ammonium acetate in various conditions.

If this poisoning is followed on a time basis, in the liver, acetylaspartic acid is almost absent at 0 time and its concentration increases to reach a peak at death. If on the contrary, a protective dose of arginine is administered prior to the lethal dose of ammonium acetate, the concentration of acetylaspartic acid increases, reaches a maximum, and slowly decreases to a normal level (Table II). Examination of the results in Table II also shows that the concentration of acetylaspartic acid is correlated with aspartic acid, whether the animals are destined to survive or to die. However, it is not correlated with NH_3 and urea. It is interesting to speculate on the origin of acetylaspartic acid. For instance, it is probable that acetylaspartic acid production *in the brain* is not geared to increasing concentrations of ammonia in that tissue; as mentioned earlier in this study and as reported in another work (3), although the concentration of acetylaspartic acid in the brain is high under normal conditions,

TABLE II

Some liver metabolites in ammonia-poisoning

Time (min) after injection of an $LD_{99.9}$ dose of NH_4Ac	Metabolites injected 1 hour prior to the $LD_{99.9}$ dose of NH_4Ac															
	None				1 mM/kg L-arginine.HCl				4 mM/kg L-arginine.HCl				1 mM/kg L-ar.HCl + 4 mM/kg NaCl			
	NH_3 N	Aspartic	Acetyl-aspartic†	Urea N	NH_3 N	Aspartic	Acetyl-aspartic†	Urea N	NH_3 N	Aspartic	Acetyl-aspartic†	Urea N	NH_3 N	Aspartic	Acetyl-aspartic†	Urea N
	Concn. of some metabolites in the liver (mg%)															
0	2.3	20.0	0.0	17.0	2.3	10.0	0.0	14.0	2.0	31.0	0.0	22.0	5.1	47.0	0.0	19.8
5	10.6	170.0	0.4	11.4	14.4	90.0	1.8	14.6	9.1	48.0	0.6	46.2	12.5	94.0	2.1	18.9
10	16.	218.0	—	36.0	18.0	81.0	4.0	16.9	6.3	33.0	0.6	55.	12.8	141.0	0.7	26.6
15*	14.	207.0	2.7	20.0	13.5	102.0	2.2	32.7	6.3	230.0	2.4	63.	13.8	99.0	3.7	28.2
30									4.1	19	1.6	53.3	5.3	28.0	0.3	35.5
60									1.4	18	0.0	50.	2.1	13.0	0.0	27.6

*The animals that received an $LD_{99.9}$ dose of NH_4Ac either alone or with a metabolite that did not protect against ammonia toxicity usually died after 15 minutes.
†Note the relationship between aspartic acid and acetylaspartic acid whether the animal is destined to survive or to die.

it does not increase in ammonia poisoning. However, acetylaspartic acid in the liver depends directly or indirectly on the presence of ammonia in that tissue. By "directly" we mean that acetylaspartic acid could be an intermediate in the Krebs–Henseleit urea cycle. This is highly improbable because there is no correlation between the concentration of acetylaspartic acid on one hand and ammonia and urea from another hand. By "indirectly" we mean that it is possible that acetylaspartic acid concentration would increase merely as a result (through the law of mass action) of the tremendous levels of aspartic acid obtained in ammonia poisoning. We are inclined to favor the second possibility because acetylaspartic acid is correlated with aspartic acid in ammonia poisoning.

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SOME ASPECTS OF THE ENZYMIC HYDROLYSIS OF URINARY 17-KETOSTEROID CONJUGATES[1]

R. HOBKIRK AND J. J. COHEN

Abstract

Four enzyme preparations containing β-glucuronidase, of bacterial, mammalian, and molluscan origin, have been shown to be equally effective in liberating 17-ketosteroids (17-KS) of the 5β-(etiocholane) configuration in normal urine. The bacterial preparation releases steroids of the 5α-(androstane) configuration more rapidly than do the molluscan enzymes and with much greater ease than does the liver enzyme. In view of the data obtained it seems unlikely that the striking difference between the bacterial and liver enzymes can be due to the hydrolysis of some labile conjugate, such as sulphate, by the former and not by the latter. Possibilities that the difference is due to the hydrolysis of an unknown type of urinary conjugate by the bacterial preparation, or to the low specificity of the bacterial β-glucuronidase, are discussed. The high degree of hydrolysis of 17-KS conjugates by the bacterial enzyme followed by solvolysis suggests this as a most useful hydrolytic procedure.

Introduction

It is now generally recognized that the measurement of individual neutral urinary 17-ketosteroids (17-KS) as a test of endocrine function is much superior to the group assay of total 17-KS (1–6). In order to separate and measure these steroids, which are present in urine as glucuronide and sulphate conjugates, a hydrolytic procedure that results in no artifact formation is required as a preliminary step (5, 7). This involves the use of enzyme preparations from various sources containing β-glucuronidase and in some cases sulphatase (8–12). Quantitative hydrolysis of urinary 17-KS sulphates by sulphatases is unlikely, because of the inhibitory effect of certain ions on these enzymes and to stereochemical considerations (13–15), but these labile conjugates are conveniently split by certain mild acid treatments (7, 16).

It has been reported that mammalian liver β-glucuronidase was unable to liberate the same amounts of 17-KS from urine as did hot acid hydrolysis (17), especially where steroids of the 5α-(androstane) configuration were concerned. An extract from the snail *Helix pomatia* did not show this difference to such an extent (18), and an enzyme preparation from the limpet *Patella vulgata*, together with mild acid hydrolysis, has been shown to liberate a very high percentage of urinary 17-KS (5, 19).

To our knowledge, a detailed investigation of the liberation of individual urinary 17-KS by a bacterial enzyme preparation has not been reported upon. A study has therefore been made comparing the ability of four enzyme preparations of bacterial, mammalian, and molluscan origin to hydrolyze urinary 17-KS conjugates. Special attention has been paid to the hydrolysis of conjugated 5α-17-KS by the bacterial and mammalian liver enzymes. The steroids measured were the three main C19 11-oxygenated metabolites of adrenocortical

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steroids, 3α,11β-dihydroxyetiocholan-17-one (11β-hydroxyetiocholanolone),* 3α,11β-dihydroxyandrostan-17-one (11β-hydroxyandrosterone) and 3α-hydroxyetiocholane-11,17-dione (11-ketoetiocholanolone), together with the 11-deoxy-steroids 3α-hydroxyetiocholan-17-one(etiocholanolone) and 3α-hydroxyandrostan-17-one (androsterone), which are metabolites of both adrenal and testicular origin. Beside these, urinary 3β-hydroxy-Δ5-androsten-17-one(dehydroepiandrosterone), which is probably of adrenal origin, was measured.

Materials and Methods

Urine Collection

Complete 24-hour specimens were collected from normal males and females. Collections were made in clean polyethylene bottles containing no preservative. Three urine pools were prepared, one from female urine, the others (male urines 1 and 2) from different male specimens. Three other 24-hour male urines were used individually (male urines 3, 4, and 5). All urines were kept in the deep-freeze until analyzed.

Chemicals

Organic solvents, with the exception of ethanol, were Reagent Grade. Suitable purification was carried out where necessary. Girard Reagent-T (Brickman Co.) was used as supplied. *m*-Dinitrobenzene was purified by an accepted procedure (20). Aluminum oxide (Woelm, almost neutral, grade I) was adjusted to contain 4.5% by weight of water. All other reagents were Analytical Reagent Grade and were used as supplied.

Enzyme Preparations

Bacterial β-glucuronidase — Sigma Chemical Co.

Mammalian liver β-glucuronidase (Ketodase) — Warner Chilcott Laboratories Inc.

Molluscan β-glucuronidase (Glusulase), also containing sulphatase, prepared from the snail *Helix pomatia* — Endo Laboratories Inc.

Molluscan β-glucuronidase, also containing sulphatase, prepared from the common limpet *Patella vulgata*; powder B of Dodgson and Spencer (10).

The unit of activity of these preparations was based on their β-glucuronidase contents and was defined as that amount of enzyme which liberated 1 μg of phenolphthalein from a 0.01 *M* solution of phenolphthalein glucuronide when incubated for 1 hour at 37° C. The pH of the medium was 6.5 for the bacterial preparation, 5.0 for the liver enzyme, 5.2 for the snail preparation, and 4.6 for the limpet material.

Hydrolysis and Extraction of the Steroids

Volumes of urine containing 2–3 mg of total 17-KS, as measured after hot acid hydrolysis, were incubated for 12-, 48-, and 96-hour periods at 37° C with each of the enzyme preparations. Conditions of pH and enzyme concentrations are given in Table I. For the bacterial and snail enzymes urine pH was adjusted

* For the sake of convenience the following abbreviations in nomenclature are used in this paper: 11β-hydroxyetiocholanolone, 11-OH-E; 11β-hydroxyandrosterone, 11-OH-A; 11-ketoetiocholanolone, 11-KE; etiocholanolone, E; androsterone, A; dehydroepiandrosterone, DHA.

with dilute NaOH or glacial acetic acid (18, 21). Acetate buffer was used for the other two preparations (3, 5). The enzyme concentrations in Table I were those found to achieve apparently maximum release of total urinary 17-KS after incubation for 48 hours with normal urine.

Following incubation, neutral ether extracts were prepared in readiness for Girard separation. Solvolytic cleavage of labile 17-KS conjugates, e.g. sulphates, was performed on the urines both before and after enzyme hydrolysis. In the latter case, urine, after extraction of 17-KS liberated by the enzymes, was made 2 *N* with 50% H_2SO_4, the subsequent steps being those originally described (16).

Where hot acid hydrolysis was performed following solvolysis the acidified urine from the latter step, together with the alkaline washes of the ether extract from the enzyme-hydrolyzed urine, was refluxed for 1 hour on a steam bath.

All analyses were performed in duplicate.

Separation and Measurement of 17-KS

The dried extracts were separated into ketonic and non-ketonic fractions by a modification of the Girard reaction (22) performed at room temperature. The ketonic fractions were then separated into their six main 17-KS by alumina column and paper chromatography, essentially as described by Brooks (19). Steroid containing zones on the paper chromatograms were eluted and analyzed by a modified Zimmermann reaction (23), reading optical densities at 480, 520, and 560 mμ on a Beckman Model B or Unicam SP600 spectrophotometer. Allen's equation (24) was used to correct for non-specific chromogens. Each steroid was initially measured in terms of DHA and then corrected for the appropriate extinction coefficient. Portions of the ethanolic eluates from paper chromatograms were treated with concentrated H_2SO_4 and scanned between 220 and 600 mμ (25) on the Beckman Model DK-2 recording spectrophotometer. The spectra were compared with those obtained using pure steroids.

Reliability of the Method

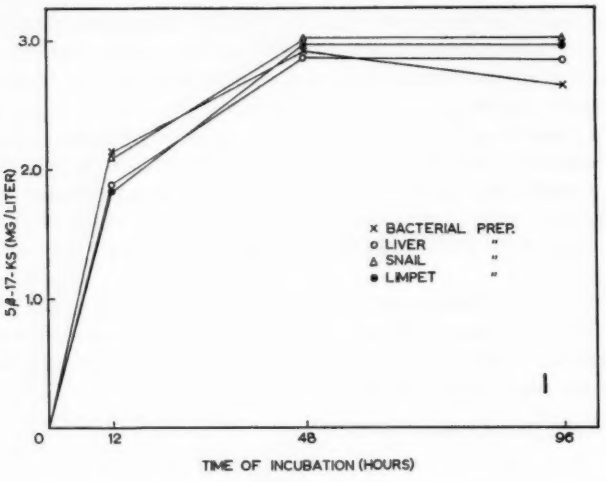
Recovery of pure steroids added to hydrolyzed urine and taken through the whole procedure was 87% $\pm$11 (standard deviation).

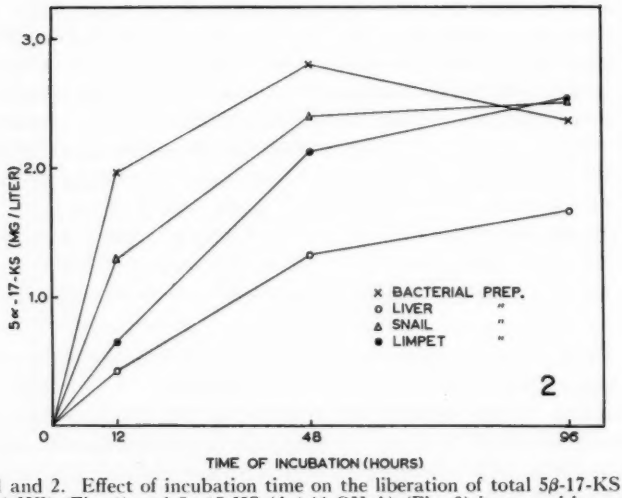
The precision of the method was assessed by calculation of an estimate of the standard deviation (s) from the difference of duplicate determinations in a series of measurements s being $\sqrt{\{S\ (d^2)/N\}}$ where d is the difference between duplicates and N is the number of duplicate analyses performed (26). For the range 0.10 – 0.99 mg of any 17-KS/liter of urine, $s = 0.076$, while for the range 1.00–4.00 mg/liter, $s = 0.20$. The lower limit of sensitivity of the method is 0.15 mg of any one 17-KS/liter of urine.

Results

Release of 5α- and 5β-17-KS by Enzymes

Figure 1 shows total 5β-17-KS (E + 11-OH-E + 11-KE) liberated in normal female urine during incubation for 12, 48, and 96 hours with each of the four enzyme preparations. Good agreement for the different enzymes existed at each time and the same was true for the individual 5β-17-KS (not shown).





FIGS. 1 and 2. Effect of incubation time on the liberation of total 5β-17-KS (E+11-OH-E+11-KE) (Fig. 1) and 5α-17-KS (A+11-OH-A) (Fig. 2) in normal human female urine by β-glucuronidase-containing preparations of bacterial, mammalian liver, and molluscan origin.

However, the bacterial preparation caused much greater hydrolysis of conjugated 5α-17-KS (A + 11-OH-A) in the same urine than did the liver enzyme (Fig. 2) under the experimental conditions employed. The bacterial preparation also achieved a more rapid release of these steroids than did the snail and limpet enzymes (Fig. 2, Table I). A tendency for 17-KS levels to decrease when incubated for 96 hours with bacterial enzyme may have been due to bacterial destruction since no antibiotics were used. In each of three separate urine pools about two and five times more A and 11-OH-A, respectively, were released by

TABLE I

Release of urinary androsterone by enzymes, results expressed as mg/liter of urine

Enzyme	Female urine			Male urine 1		
	Time of incubation (hours)					
	12	48	96	12	48	96
Bacterial, pH 6.5, 75 units/ml	1.70	2.25	1.80	2.34	2.53	1.40
Liver, pH 5.0, 300 units/ml	0.35	1.20	1.45	0.25	1.04	1.40
Snail, pH 5.2, 500 units/ml	1.20	2.20	2.20	1.07	1.95	2.67
Limpet, pH 4.6, 750 units/ml	0.50	1.85	2.20	0.74	1.57	1.99

75 units/ml of bacterial preparation than by 300 units/ml of liver enzyme (Table III). An increase in liver enzyme concentration to 750 units/ml still failed to equal the performance of the bacterial enzyme. E and 11-OH-E levels were not significantly different in these urines regardless of whether bacterial or liver enzymes were employed.

Hydrolysis of Labile 17-KS Conjugates

Minimal amounts of DHA (if any) were freed by bacterial and liver enzymes (Table II) while the snail and limpet preparations hydrolyzed slightly more. This might be expected because of the sulphatase content of the latter two.

TABLE II

Liberation of DHA in normal urine;* results expressed as mg/liter of urine

Enzyme†	Free DHA after incubation with enzymes		DHA liberated by solvolysis following incubation with enzymes		Total DHA (sum of previous two columns)	
	Male urine 1	Female urine	Male urine 1	Female urine	Male urine 1	Female urine
Bacterial	0.21	0	1.33	0.32	1.54	0.32
Liver	0.24	0	1.55	0.32	1.79	0.32
Snail	0.38	0.12	1.22	0.18	1.60	0.30
Limpet	0.50	0.29	0.86	(?)	1.36	0.29

*Solvolysis of the original urine before enzyme action showed male urine 1 and the female urine to contain 1.80 and 0.34 mg DHA/liter, respectively.
†Conditions of incubation as in Table I; time of incubation, 48 hours.

Virtually all the urinary DHA (as measured prior to enzyme action) was liberated by solvolysis of the urine after incubation with bacterial or liver preparation. Of the other 17-KS measured only E and A were detected in the free form after solvolysis of the original urine from both males and females. Out of 0.21 mg of A measured in this way in a female urine pool, 0.18 and 0.22 mg remained to be freed by solvolysis following 48 hours' incubation with bacterial and liver enzyme respectively. Corresponding results for a male urine pool were 1.03 mg of A before enzyme action with 0.72 and 0.92 mg released by solvolysis after bacterial and liver enzyme action. These results are interpreted as meaning that the activities (if any) of the bacterial and liver enzymes towards labile 17-KS conjugates are similar.

TABLE III

Liberation of urinary 17-KS by bacterial and mammalian liver enzymes in 48 hours; results are expressed as mg/liter of urine*

	Female urine			Male urine 1			Male urine 2			
Enzyme†	E	A	11-OH-A	E	A	11-OH-A	E	11-OH-E	A	11-OH-A
Bacterial	2.45 (2.05–2.85)	2.25 (1.85–2.65)	0.56 (.41–.71)	2.25 (1.85–2.65)	2.53 (2.13–2.93)	1.28 (.88–1.68)	3.20 (2.80–3.60)	0.40 (.35–.55)	4.23 (3.83–4.63)	1.29 (.89–1.69)
Liver	2.50 (2.10–2.90)	1.20 (.80–1.60)	0.10 (0–.25)	2.64 (2.24–3.04)	1.04 (.64–1.44)	0.26 (.11–.41)	2.88 (2.48–3.28)	0.30 (.15–.45)	2.40 (2.00–2.80)	0.29 (.14–.44)
Liver (750 units/ml)		—			—		3.34 (2.94–3.74	0.43 (.28–.58)	3.27 (2.87–3.67)	0.63 (.48–.78)

*Fiducial range ($p = 0.01$) is shown in parentheses for each result.
†Conditions of incubation as in Table I except where indicated.

Hydrolysis of Total 17-KS Conjugates

Complete liberation of 17-KS in normal human urine was achieved by incubation for 48 hours with bacterial enzyme followed by solvolysis. Table IV gives the levels of six 17-KS fractions freed by this procedure. Hot acid hydrolysis after solvolysis resulted in a small positive measure for total 17-KS but in no case was a typical Zimmermann-positive zone seen on paper chromatograms of these extracts.

TABLE IV

Amounts of individual 17-KS released in normal male urine by bacterial enzyme followed by solvolysis;* results expressed as mg/24 hours

Urine specimen	Age (years)	DHA	E	A	11-OH-E	11-OH-A	11-KE
Male urine 3	22	0.59	2.96	4.79	0.31	0.88	0.45
Male urine 4	22	2.10	2.68	3.47	0.58	1.29	1.00
Male urine 5	55	0	0.89	0.72	0.20	0.65	0.46

*Individual 17-KS were not released in measurable amount by further hot acid hydrolysis.

Discussion

Incomplete hydrolysis of 5α-17-KS conjugates by mammalian liver β-glucuronidase has been reported by Wotiz *et al.* (17). In order to explain their findings these workers suggested the presence of non-glucuronide conjugates (possibly sulphates) in the urine. However, separation of 17-KS glucuronide and sulphate fractions from urine has shown little or no 11-OH-A to be present as the latter type of conjugate (5, 19, 27), which is in agreement with the results in the present work. Also, any likelihood that the difference in activity between the bacterial and liver enzymes might be due to sulphatase activity in the former is further diminished by the finding that neither of these preparations liberated much of the DHA or A present as labile conjugate. Phosphatase activity has been shown to occur in the snail enzyme preparation (28), and a complex containing DHA and phosphate has been identified in plasma (29). If steroid phosphates were present in the urine, however, they would presumably constitute part of the labile conjugate fraction. Therefore any possibility that phosphatase might play an important part in the bacterial enzyme activity could be dismissed on the same grounds as for the sulphatase. An alternative explanation would be to postulate the occurrence of a hitherto unrecognized 5α-17-KS conjugate, of a non-labile nature, capable of being hydrolyzed by a similarly hypothetical enzyme constituent of the bacterial preparation. Experiments to test this possibility are at present being conducted in this laboratory. The existence of a urinary conjugate of 11-OH-A, resistant to hydrolysis by enzyme or mild acid treatment, has been reported (7). Although inhibitors of β-glucuronidase (30, 31) in urine might be considered to account for the lower activity of the liver preparation, the preferential inhibition of 5α-17-KS conjugate hydrolysis would still remain to be explained.

Besides these possible explanations it is conceivable that the bacterial β-glucuronidase is generally a less specific enzyme than the mammalian liver one and therefore able to achieve more rapid hydrolysis of a greater variety of

glucuronides. That the action of the bacterial enzyme on 17-KS conjugates is basically a manifestation of glucuronidase activity is supported by the essentially complete hydrolysis of urinary conjugates, primarily of the 5α-17-KS type, by a sufficiently long incubation with the purified liver β-glucuronidase (31, 33). The complete hydrolysis achieved by the bacterial enzyme plus the solvolytic procedure, together with the good agreement in individual 17-KS levels obtained when compared with the results of other workers (3, 5, 19) suggests this as a useful hydrolytic method.

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THE RELATIONSHIP BETWEEN ELECTRICAL AND MECHANICAL ACTIVITY OF THE SMALL INTESTINE OF DOG AND MAN[1]

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Abstract

Electrical activity of the small intestine of man and of dogs has been studied using monopolar recording techniques and spread of electrical activity in the small intestine of the dog using a bipolar recording technique. Motility was studied simultaneously. Electrical activity consisted of slow waves and action potentials which occurred when contractions were present. Action potentials were not conducted but slow waves sometimes spread aborally for short distances. Particular attention was paid to the relation of slow waves to action potentials and to motility. No consistent alteration in the frequency or configuration of slow waves was found associated with the occurrence of action potentials and motility, although serotonin or epinephrine altered slow wave frequency slightly. Slow waves usually were increased in amplitude during periods when motility and action potentials were occurring (during eating or balloon propulsion; after the administration of serotonin, neostigmine, physostigmine, or morphine). Slow wave amplitudes usually were diminished when motility was inhibited (by balloon distention; after administration of epinephrine, etc.). Action potentials tended to occur in phase with the slow waves, when the muscle electrode was positive relative to the indifferent electrode, but this was not always so during non-propulsive contractions. There was also a correlation between the occurrence of distal spread of slow waves over the duodenum and upper jejunum and the ability of the intestine in this region to respond to balloon distention by propulsion.

In the dog, body temperature consistently affected slow waves. A decrease of 10° C diminished their frequencies to less than one-half and diminished their amplitude. Slow waves occurred at similar frequencies and with regular conduction after large doses of nicotine or atropine. Dibenzyline, dichloroisopropylnorepinephrine, and vagotomy did not markedly alter slow wave frequencies. These findings and those in our studies with microelectrodes indicate that the slow waves are myogenic in origin, and represent electrical currents in the extracellular fluid initiated by periodic depolarizations of muscle cells of the small intestine.

A number of investigators have recorded the electrical activity of the small intestine (1–11) of dog and other species. In all instances both slow and fast electrical waves have been recorded. Both types of activity appear to originate from the muscular layers of the small intestine (10, 11). There is general agreement that contractile activity is associated with the fast electrical waves, which, when recorded with sufficiently small electrodes, appear to correspond to action potentials. A body of evidence suggests that these action potentials do not spread more than 1 to 2 mm (10–13). The relation of slow electrical waves to intestinal function is less well understood. Although bursts of action potentials usually occur in phase with slow waves, they may occur independently (11). In addition, slow waves persist in the absence of mechanical activity, for example, in the relaxed intestine of an atropinized animal (7, 10, 11). Intestinal slow waves occur with diminishing frequency at more distal regions of the small intestine (from 17–22/minute in the duodenum to 9–12/minute in the terminal ileum) (2, 3, 7–11). The velocity of aboral spread of these waves over short distances also have been reported to be diminished in

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more distal portions of the small intestine (8). The hypothesis (9) that slow wave frequency of the intestine is determined by a pacemaker located in the duodenum near the ampulla of Vater has been advanced despite the difficulties in explaining the different frequencies of different intestinal segments. This hypothesis has not been confirmed in studies in this laboratory (11). The physiological value of a single pacemaker seems dubious since the mechanical activity of the intestine must be adapted to the local content of intestinal material and so must be partially under local control (15, 16).

The present study was designed to answer the following questions: (1) What changes occur in slow waves during procedures which induce or inhibit motility? (2) What changes are produced in slow waves by the various naturally occurring intestinomotor substances? (3) What changes are produced in slow waves by pharmacological agents which block the actions of these substances? It was hoped that these studies might provide information both as to the role of slow waves in intestinal physiology and to their origin.

Methods

Recording of Electrical Activity. External Wire Electrodes

The method developed for studies in dogs has been described previously (11). Briefly, electrodes of thin silver wire (0.006 in. in diameter) were sewn into the intestinal wall under the serosa. All but the terminal 1.5 cm of the electrode was insulated and the electrode site was insulated from surrounding intestinal segments. The method for insulation of the electrode sites in the present study was modified by using gauze pads impregnated with liquid paraffin. This modification did not result in any alteration in the nature of the slow waves recorded but allowed continuous undisturbed recordings for many hours without replacement of the gauze pads. The usual sites chosen for placement of the electrodes were in the duodenum below the opening of the bile duct, in the upper jejunum 5–10 cm below the ligament of Treitz, and in the ileum about 10 cm proximal to the ileocecal junction. After the electrodes were in place, the leads were brought out through the incision in the abdominal wall and the incision was closed. The leads from the electrodes were connected to a selector switch interposed between them and the C lead of the Sanborn Visocardiette. This selector switch enabled the leads from the various portions of the intestine to be connected, one at a time, to the C lead. The circuit was completed by attaching a ground electrode to the left leg and to the left leg lead on the Visocardiette and by placing the selector switch of the Visocardiette to the CF position. A sensitivity of 1 cm per millivolt was used unless otherwise specified. The paper speed was 2.5 cm/second. One centimeter on the paper corresponds to 2 of the large divisions or to 10 of the small divisions. Determinations of slow wave frequency were made by taking the time for 10 slow waves and converting this into a figure for frequency. Timing of the slow wave frequencies in these experiments was based on the end of the rapid upward (positive) deflection which was usually completed in less than 100 milliseconds. In addition, the temporal relations between action potentials and slow waves were made using this point in the slow wave as zero time. The measurements

could not have been significantly in error as a result of the fact that the recording system had a time constant of 2 seconds.

In the studies in patients with ileostomies the silver wire electrodes were of the same size as those used in the animal experiments. They were sterilized in zephiran solution. To aid in their insertion a No. 23 needle was inserted through the outer reflected muscle layers of the ileal bud, the electrode was threaded into the needle tip, and the needle withdrawn leaving the electrode in place. The indifferent electrode was placed parallel to the long axis of the intestine on the mucosa near the base of the ileal bud.

Studies of Motility in the Dog Intestine

A condom balloon (3.5 cm long) was fitted to a polyethylene catheter, which was marked off in centimeters starting from the tip of the balloon. The balloon was inserted into a tiny stab wound made just beyond the pyloric sphincter. Bleeding was controlled with Gel-foam. The distance between this wound and the duodenal electrodes were then recorded. Another stab wound was made through the body wall beside the xiphisternum, and the polyethylene catheter was withdrawn through this wound. Care was taken to ensure that friction between the catheter and the wounds in the body wall and in the duodenum was negligible, so that the balloon was free to move through the duodenum. The balloon was then withdrawn until it was just inside the duodenum and the length of catheter protruding from the body when there was no slack was noted. The abdominal incision was then closed. The balloon was inserted 1–2 cm further into the duodenum and inflated with from 5 to 7 ml of air. Its movement through the duodenum was followed by noting the length of catheter, in centimeters, protruding from the body wall. This method is reliable only for the duodenum, which is anchored by its mesenteric attachments. The reliability of this method was checked in several ways. The same length of catheter invariably protruded when the balloon was deflated and drawn back to the duodenal wound. In addition, occasional checks of the actual position of the balloon against its estimated position were made and found to be accurate within 1 cm. Finally, while recording was done from a given electrode, action potentials always appeared at a constant balloon position. When action potentials first appeared, the middle of the balloon was calculated to be just distal to the electrode.

Conduction Studies

The spread of intestinal slow waves was studied by the following procedure. Two electrodes were placed at varying distances apart (usually 6 cm but up to 40 cm) in the upper small intestine. They were attached to the input of the Sanborn Visocardiette (C and LL leads) and the selector switch turned to the CF position for recording. If conduction was occurring, this resulted in the appearance of a regular biphasic wave in the record. The rate of slow wave propagation was determined by taking the time interval between the two peaks in the record and dividing it into the interelectrode distance.

Drug Injections

Rapid injections

Both long- and short-acting drugs were sometimes administered in a rapid intravenous injection. This method was satisfactory with long-acting drugs. With drugs of short duration of action an attempt was made to follow the intestinal electrode activity until all the effects of the injection had subsided and to correlate the electrical recordings with the various phases of drug action.

Infusions

To allow effects of short-acting drugs to be studied with more constancy, infusions of these agents were made by means of a multiple-vane milking-type infusion pump which provided a pulsatile flow. Over time intervals of more than a few seconds, the output of the pump per unit time was constant.

The drugs were administered in terms of the salts unless otherwise stated.

Results

I. Studies in Dogs

A. Monopolar Recordings

1. Balloon Distention

Distention by a balloon has long been known to excite the intestine proximal to the balloon and to inhibit the intestine over the balloon and distal to it (15, 16). If the balloon is not too large this results in its propulsion. During initial experiments using pentobarbital anaesthesia the dominance of sympathetic tone (tachycardia, hypertension, intestinal atony) prevented propulsion of even small balloons. The use of chloralose anaesthesia (after induction with divinyl ether) resulted in a slow heart rate, a normal blood pressure, and an intestine responsive to distention.

More than 50 records were taken in which a balloon was propelled past an electrode placed in various parts of the duodenum. Table I summarizes a number of these. Figure 1*b* (p. 794) illustrates a typical record. Some variation in slow wave frequency occurred in such records but it was inconsistent and in many records such as shown in Fig. 1*b*, no variations in frequency were found.* In almost every instance the occurrence of excitation at the electrode, signalled by the appearance of action potentials, was accompanied by an increase in the magnitude of the slow waves (also see Table I). In this record, as in other instances of propulsive motility, the action potentials were in phase with the slow wave and occurred when the muscle electrode was positive relative to the indifferent electrode. Before the appearance of action potentials no consistent alteration in slow wave amplitudes was noted.

2. Effects of Procedures and Drugs which Induce Motility

Serotonin.—Serotonin was given either in a rapid injection or by continuous slow infusion. Following the intravenous administration of varying quantities of serotonin by a rapid injection, there was usually an initial burst of action potentials in a few seconds after the injection. This was followed by larger

*In many of those instances in which there was variation in the frequency of the slow waves, the change was associated with an alteration in slow wave pattern. This consisted of the appearance of slow waves initiated by a sharp negative deflection, which occurred at a slower rate.

TABLE I

Slow wave changes during excitation by a distended propelled balloon in the dog duodenum and upper jejunum

	Slow wave maximum amplitudes* (mv)			Slow wave frequency* (min^{-1})		
	Control	Before action potentials	During action potentials	Control	Before action potentials	During action potentials
	1.56(24)	1.50(24)	3.45(29)	18.5(24)	18.6(24)	18.8(28)
Range	0.5–4.5	0.5–3.7	0.7–12.5	14.7–22.5	14.5–22.8	14.4–24.6
Comparison	1 vs. 3	2 vs. 3		1 vs. 3	2 vs. 3	
P†	<.01	<.01		>0.5	>0.5	

*The values used were the maximum amplitude and the average frequency during the intervals studied.
†P is the probability that the difference indicated was due to chance alone.

slow waves and by intermittent action potentials in phase with them. Gradually thereafter the record returned to the control state. There was a gradient of sensitivity to the drug in the intestine in that action potentials were often recorded from the duodenum when none were recorded from the ileum. Serotonin was infused at various rates from 10 to 200 μg/kg/minute in four dogs. Typical results are summarized in Table II and illustrated in Fig. 2. The initial infusion in each animal was accompanied by action potentials in the duodenum when the drug was delivered at slow rates (10 mg/kg/minute). Sometimes action potentials occurred in the jejunum as well when rapid rates (200 μg/kg/minute) of delivery were used, but none were recorded from the ileum. Slow wave amplitudes tended to be increased when serotonin-induced

TABLE II

Effect of various procedures on slow wave frequency and amplitude in dogs

Dog	Dose or infusion rate	Intest. segment	Slow wave ampl. (mv) Control	A.P.'s present	A.P.'s gone	Slow wave freq. (min^{-1}) Control	A.P.'s present	A.P.'s gone	Effect on conduction
			Procedure: Injection of physostigmine						
1	2.5 mg I.V.	U.D.*	1.0	2.7–3.2	1.0–1.1	17	14–15	16–19	Not followed
		L.D.	0.9	1.7	0.6–0.9	15.5	15	16–17	
		JEJ.	1.9	1.9–2.9	1.2–1.3	17	14–15	14	
		IL.	0.9	2.2–2.5	1.0	12.5	10–13.5	11–14	
			Procedure: Infusion of serotonin						
			Before	During	After	Before	During	After	
17	10 μg/kg/min	U.D.	2.7	3.4†	1.2	17.5	16	16	Conduction 12–20 cm/sec throughout
		L.D.	2.0	2.1†	1.1	17.5	16	16	
		JEJ.	4.7	3.7	4.3	17.5	15.5	16.5	
		IL.	1.0	2.6	1.2	14	12	—	
	200 μg/kg/min‡	U.D.	2.3	1.9	3.0	16	15	15	Conduction (12–20 cm/sec) failure during infusion
		L.D.	1.7	1.2	1.0	16	15	15	
		JEJ.	4.5	4.5	3.3	16	15	14.5	
		IL.	2.5	1.3	1.7	11	—	—	
			Procedure: Infusion of epinephrine						
6	10 μg/kg/min	U.D.	2.0	1.4	0.8	17.3	19	17	Conduction (9–10.5 cm/sec) failure during infusion
		L.D.	2.0	0.7	1.7	17.3	18	17	
		JEJ.	1.5	0.5	—	17	18	17	
		IL.	0.5	0.6	—	13	12	12.5	
15	10 μg/kg/min	U.D.	4.0	0.6	—	22	25	—	Conduction (13.5–14 cm/sec) failure during infusion
		L.D.	3.8	1.6	—	21	25	—	
		JEJ.	2.8	1.5	—	20	25	—	
		IL.	1.5	1.1	—	15	18	—	
			Procedure: Inhibition by balloon distention						
10–21		U.D.	1.8–2.5	0.5–1.8	1.3–2.0	15.2	15.0	14.9	Not followed
		M.D.	1.6–1.8	0.9–1.4	1.2–4.0†	20.3	18.5–19.5	18.0–18.2	
		U.D.	0.6–2.2†	0.9–1.2	0.2–1.5†	16.5	16.6	15.9	

*Abbreviations: U.D. upper duodenum, M.D. mid duodenum, L.D. lower duodenum, JEJ. jejunum, IL. ileum.
†Action potentials present.
‡Adaptation to previous serotonin infusions; no action potentials.

action potentials and contractions were occurring. Slow wave frequency decreased in response to serotonin infusion. Sometimes this persisted for up to several hours after cessation of the infusions (Table II). This persistence of slow frequencies may have been the result of decreased body temperature (see below). Serotonin had no consistent effect on the conduction of slow waves at two duodenal electrodes (Table II). The velocities of spread calculated from such records were not altered by this agent, but in several instances temporary dissociation of slow waves in the bipolar records was produced.

Adaptation to serotonin has been observed in studies of a variety of tissues which respond to this drug (17). Adaptation was also a prominent feature of the electrical response of the intestine to serotonin and complicated the interpretation of results. If infusions were continued more than 9–12 minutes, all action potentials disappeared and subsequent infusions at up to 20 times the initial rate were virtually ineffective in initiating action potentials (Table II and Fig. 2). Decrease of slow wave frequency in response to serotonin occurred irrespective of previous exposure to this agent.

Physostigmine (Eserine).—Fifteen dogs were given varying quantities of physostigmine (0.1 to 10 mg) intravenously. A portion of one such experiment is summarized in Table II. The slow wave frequency was not consistently altered by injection of effective quantities of this anticholinesterase drug. Action potentials were produced in the small intestine within a few minutes following the injection of physostigmine. As with serotonin, there was a gradient of intestinal sensitivity to the drug. The duodenum always was the most sensitive and action potentials were invariably produced, but they were less frequently recorded from the ileum. All the effects of eserine diminished markedly or disappeared after about 30 minutes.

The slow wave amplitudes were usually larger during periods when action potentials were occurring than during periods without action potentials before or after the administration of the drug. In addition, if action potentials were not produced there was usually no increase in slow wave amplitude. In addition, during periods when action potentials were present only occasionally at a given electrode, there was sometimes no clear-cut difference between the amplitudes of slow waves which were accompanied by action potentials and those which were not. There was, however, an association between the occurrence of action potentials and larger slow waves. Slow wave frequencies were sometimes diminished after large doses of physostigmine were administered but this change was unrelated to the initial presence or subsequent absence of action potentials.* This decrease in frequency may have resulted from anoxia (10), since it was accompanied by respiratory distress and by cyanosis of the intestine.

Acetylcholine and Vagal Stimulation.—Several studies were carried out in which acetylcholine was rapidly injected or infused intravenously (1–1000 μg/kg/minute) or in which the vagi were stimulated (before and after vagotomy

*The appearance of the gut in such instances always suggested a cord-like spasm even when action potentials had disappeared. If physostigmine acts to depolarize the intestinal muscle cells it may initially cause contraction with action potentials and then contracture without action potentials.

proximal to the site of stimulation) at rates of 5 to 100/second. Action potentials occurred only occasionally during slow infusions of high concentrations of the drug. Slow wave frequencies were not altered consistently, but their amplitudes increased if action potentials were initiated. Often the response to rapid injections resembled that to epinephrine (see below). Presumably, this resulted from the reflex release of endogenous epinephrine in response to hypotensive effects of these procedures.

Nicotine Sulphate.—One to five milligrams/kilogram of nicotine sulphate was injected in three animals. These doses completely blocked the cardiovascular effects of vagal stimulation. The intestinal response recorded by a balloon–kymograph technique consisted of a spasm which continued until the initial hypertensive effects of nicotine had disappeared. When no balloon was present in the intestine, action potentials were usually produced only in the duodenum. There were, however, no significant changes in frequency of slow waves during either the initial response to nicotine or during the subsequent period of ganglionic blockade. When action potentials were occurring the slow wave amplitudes were greater.

Morphine Sulphate.—Morphine (0.5 mg/kg I.V.) usually produced an increase in the amplitude of slow waves and sometimes a slight, variable decrease in their frequency. Action potentials always accompanied the spastic, nonpropulsive increase in contractile activity induced by morphine. It is noteworthy that on several occasions the action potentials occurred unrelated to the phase of the slow waves, i.e. in the negative troughs as well as on the more positive peaks (11).

Mechanical Stimulation.—Mechanical stimulation by gentle stroking of the intestine transversely to its long axis caused the appearance of a stationary contraction ring less than 1 cm in width. The subsequent behavior of this ring was similar to that described by Hukuhara *et al.* (16). There were action potentials associated with contractions which spread orally. As illustrated in Fig. 3, these were not in phase with slow waves and the slow wave frequency was diminished.

3. Effects of Procedures and Drugs which Inhibit Motility

Epinephrine.—The injection of relatively small amounts of Adrenalin hydrochloride® (1 μg/kg) caused a marked but transient inhibition of both electrical and mechanical activity (18). The inhibition of electrical activity was characterized by a diminution of slow wave amplitudes, sometimes so marked as to make calculation of frequencies impossible. In other cases, the frequency seemed to increase slightly. Action potentials, if present, were inhibited briefly and their return accompanied the first visible or recordable contraction.

Infusions of epinephrine (1–100 μg/kg/minute) usually caused diminution of slow wave amplitudes in duodenum and jejunum, but not in the lower ileum. Their frequency was increased. Aboral spread of slow waves if present was disrupted (Table II). Gradually, with continued infusion, the intestine appeared to adapt to the presence of epinephrine as it had to serotonin administered by infusion.

Effect of Distention of Balloon Placed Proximal to Electrode.—As illustrated in Table II, moderate distention of a balloon with 5–7 ml of air proximal to the recording electrode had no consistent effects on slow wave frequency. It did, however, inhibit action potentials if these were present prior to distention of the balloon (Fig. 4). The magnitude of the slow waves was usually decreased during the inhibitory effect of balloon distention on the intestine. These effects disappeared if the balloon was propelled beyond the electrode or was deflated.

Other Drugs: Atropine Sulphate.—In doses up to 5mg/kg I.V. atropine sulphate had no significant effect on slow wave frequency. It did, however, consistently diminish slow wave amplitude and simultaneously abolish action potentials and motility if these were present.

Other Drugs: Hexamethonium and Pentamethonium Bromide (1–5 mg/kg I.V.), *Dibenzyline* (5–10 mg/kg), and *Dichloroisopropylnorepinephrine* (10 mg/kg).—Following administration of these drugs no consistent effects on electrical activity of the intestine were observed. When epinephrine was administered after the latter two drugs were given, there was no clear-cut alteration in the intestinal response to epinephrine.

Surgical Isolation of Intestine.—Complete isolation of a loop of jejunum except for its mesenteric attachment did not appreciably alter slow waves unless the temperature of the segment fell.

B. Conduction Studies using Bipolar Recording

Conduction in the Upper Small Intestine

Apparent aboral conduction of slow waves occurred during a number of bipolar recordings from electrodes 2 to 40 cm apart in the duodenum and upper jejunum. The calculated conduction rates averaged about 15 cm/second and were relatively constant between a given electrode pair in a given animal. They varied from 6 to 38 cm/second when measured between electrodes in different animals. Inaccuracies in measuring interelectrode distances and differences in body temperature (see later) probably accounted for most of the variation. Conduction regularly occurred in animals anaesthesized with divinyl ether – chloralose and in these animals propulsive activity followed distention of a balloon in the duodenum. Conduction occurred in 13 of 18 animals anaesthesized with pentobarbital.

Propulsion of a balloon down the duodenum did not affect frequency of slow waves in the upper jejunum or ileum. However, when a balloon was being propelled past two electrodes in the duodenum, some effects on conduction between them were noted (Fig. 5). When the balloon was between the electrodes, conduction velocity of slow waves was decreased and normal control values were not recorded until the balloon had been propelled past the distal electrode. Individual action potentials, unlike individual slow waves, were not conducted over 2.5 cm separating the two electrodes. Lack of conduction of action potentials over more than a few millimeters was implicit in the common observation that action potentials appeared for the first time when the balloon moved onward past the electrode, a distance of only 0.5 cm.

Duodenal slow waves were not conducted to the ileum, since ileal waves were only about one-half the frequency of duodenal slow waves. Furthermore,

records made of ileal and jejunal slow waves during and after propulsion of a duodenal balloon indicated that none of the duodenal activity was transmitted further along the intestine.

None of several types of blocking agents seemed to have appreciable effects on conduction. For example, after 5 mg/kg I.V. of nicotine sulphate, conduction was found to occur at normal velocities (10–18 cm/second) not only between the upper and lower duodenum but also between the upper duodenum and the upper jejunum 38 centimeters away. A similar lack of effect was noted after injection of 5 mg/kg I.V. of hexamethonium bromide. Similarly after morphine sulphate (1.0 mg/kg I.V.) and atropine sulphate (0.5 mg/kg I.V.), conduction occurred between the upper and lower duodenum and between the lower duodenum and upper jejunum (25 cm away) at normal velocities of 12 to 18 cm/second.

C. Effect of Body Temperature

To test the possibility that the serotonin content of the intestine might affect its slow wave activity, Reserpine® (1 mg/kg I.V.) was administered 24 hours before the preparation of a dog for slow wave recording. This drug decreases the concentration of intestinal serotonin (17). The slow wave frequency was found to be much diminished. However, the rectal temperature was also decreased. Previous reports (9, 11), confirmed in our experiments, indicated that slow wave frequency falls progressively during anaesthesia. Body temperature also decreased. It seemed possible, therefore, that the causative factor in both instances was a fall in body temperature resulting from prolonged anaesthesia. Further experiments, without pretreatment with Reserpine in which dogs were cooled by placing them in a cool environment and wrapping their tongues in ice indicated that slow wave frequencies in all portions of the intestine were directly related to body temperature. In addition,

TABLE III

Effect of body temperature on slow wave frequency (min^{-1})

Dog	Procedure	Rectal temp., °C	Upper duodenum	Lower duodenum	Jejunum	Ileum
24-9-58	Prolonged anaesthesia	35	15.7	16.4	15.7	9.9
		31	11.5	11.3	12.7	Flat record
6-7-59	Reserpine 1 mg/kg; cooled	37.0	18.0	18.0	17.0	10.5
		35.0	13.0	16.0	13.5	7.5
		33.0	12.0	11.5	8.0	7.5
		31.0	10.0	10.0	10.0	5.0
		29.0	8.0	9.0	7.5	5.0
		27.0	8.0	8.0	7.0	5.0
24-5-57	Cooled with ice and rewarmed	36.2	17.3	17.2	16.5	12.5
		31	13.6	10.2	11.7	Flat record
		29	8.0	7.7	8.3	Flat record
		27.5	7.1	—	—	—
		31.5	14.2	13.3	13.9	—
1-6-57	After reserpine 1 mg/kg; warmed	39	21.1	21.2	21.6	—
		40	22.5	22.2	21.0	15.6
		41	—	—	—	14.3
23-9-58	Effect of dinitrophenol 5 mg/kg	36.5	16.6	16.1	16.0	10.9
		37.5	—	19.3	19.6	12.5
		40.0	20.2	20.9	20.9	13.9

in other experiments in which body temperature was raised by heating or by the administration of dinitrophenol (1–5 mg/kg I.V.) slow wave frequencies of the small intestine were increased above control values. The results of some of these experiments are summarized in Table III.

Unfortunately, lacking a direct measure of the temperature of each segment, it was not possible to make a precise correlation between the temperature of an intestinal segment and its slow wave frequency. A decrease of body temperature by 10° C diminished slow wave frequencies to less than half the original value. Lowering the body temperature to below 30° C not only decreased slow wave frequency but also markedly decreased slow wave amplitudes. Duodenal conduction also diminished in velocity in one such experiment: 20 cm/second at 40°; 13 cm/second at 38°; 8 cm/second at 36°; about 5 cm/second at 30 to 31° C, and thereafter it failed. Most commonly conduction failure occurred at higher body temperatures so that the effects of temperature could not be evaluated.

II. Studies in Patients with Ileostomies

The electrical activity of the muscle of the human terminal ileum was studied on 16 occasions in two patients. The reproducibility of the method used was indicated by the small variability in the initial control values for slow wave frequency and amplitude (Table IV). That no contractions were observed and no action potentials were present in the control records taken a few minutes after the electrodes were placed, suggested that the method was innocuous.

TABLE IV

Control values for slow wave amplitude and frequency in the human ileum

Patient	No. experiments	Slow wave frequency (min^{-1})	Slow wave amplitude (mv)	
			Min.	Max.
Cr.	5	7.6 ±0.3 s.e.	0.4 ±0.09 s.e.	0.95±0.16 s.e.
Co.	8	7.25±0.3	0.34±0.08	0.83±0.14

1. *Effect of Eating*

Five experiments were performed in which the only variable was the ingestion of food after a 8- to 12-hour fast. The results of one experiment are illustrated in Fig. 6. Invariably the ileum became active 2 to 10 minutes after the patient began to eat. The slow wave frequencies were not consistently altered, but the amplitudes were generally increased and action potentials appeared in the record just preceding visible contractions at the electrode. Simultaneous balloon–kymograph recordings indicated forceful contractions and repeated evacuations occurred. Propulsive motility in the human ileum was therefore accompanied by the same kinds of alterations in the electrical record as in the dog duodenum.

2. *Effect of Drugs Inducing Intestinal Motility*

Serotonin.—The electrical response of the human ileum to serotonin has been described in detail elsewhere (19). As illustrated in Fig. 7, the occurrence

of action potentials was accompanied by an increase in slow wave amplitude.

Morphine and Neostigmine.—Both morphine sulphate (16 mg I.V.) and neostigmine methyl bromide (0.5 mg I.M.) caused an increase in the amplitude of ileal slow waves and the appearance of action potentials accompanying contractions at the muscle electrode (Fig. 7). No consistent alteration in slow wave frequency accompanied these changes.

Atropine.—Atropine sulphate (1.3 mg) was administered by intravenous injection on several occasions. Sometimes it reduced slow wave amplitudes but this was not a consistent finding. However, in almost all instances the intestine was inactive before the atropine was administered so that little additional effect was to be expected. There were no consistent effects on slow wave frequencies.

Discussion

Origin of Intestinal Slow Waves

The slow waves of the dog small intestine, like the action potentials, are believed to be muscular in origin for several reasons (10, 11). Recent studies in this laboratory with microelectrodes have shown that they correspond to slow periodic depolarizations of longitudinal muscle cells (20). The microelectrodes used did not penetrate to the circular muscle so that the possibility that a different type of electrical activity may occur in cells of this layer has not been eliminated. However, previous studies (11) in which electrical activity was recorded at various depths in the intestinal wall with macroelectrodes revealed no indication of any other periodic type of electrical activity originating from any layer of the small intestine.

The data from the present studies with macroelectrodes and from the study with microelectrodes (20) suggest that periodic depolarizations are a fundamental property of smooth muscle cells of the dog intestine. The rate of occurrence of depolarizations is dependent on temperature (11), on the anatomical location of the tissue (1–11), and on the maintenance of oxidative processes (10, 20).

In the human intestine, slow waves recorded with macroelectrodes show similar properties to those of the dog intestine insofar as they have been studied. They occur with a higher frequency in the duodenum (18–22/minute) (10, 14) than in the terminal ileum (5–8/minute) (10, 11). The effects of temperature and oxidative processes on the slow waves of the human intestine have not been studied.

Slow waves have also been recorded from the small intestine of the cat in vivo (11) and from the small intestine of rodents in vitro (4, 6). Similar waves have been recorded with microelectrodes from relatively undisturbed rabbit and guinea pig taenia coli in vitro (21, 22). It seems permissible then to propose that slow waves corresponding to periodic depolarizations of the muscle cells are the characteristic activity of longitudinal muscle of the mammalian small intestine and possibly of the circular muscle as well. Failure to record these waves from the circular intestinal muscle of the cat in recent studies with microelectrodes may have resulted from the drastic preparation of the tissue (23, 34).

Slow Waves and Motility

Slow waves occur in the relaxed as well as in the contracting intestine and at similar frequencies at a given electrode. During propulsive activity, action potentials occur during that phase of the slow wave when the muscle electrode is positive relative to the indifferent electrode. Occasionally during a drug-induced spasm or during the response to mechanical stimulation, action potentials appear in the negative troughs of the slow waves. The action potentials are usually negative-going deflections at the muscle electrode, as has previously been reported (10, 11). They were not propagated to areas only 1 cm from the site of visible contractions. In most cases, during intervals when propulsion and action potentials are occurring, the voltage change during slow waves (from positive peak to negative trough) is increased. Conversely, during inhibition of motility, slow wave voltage (amplitudes) tend to be reduced. There was often no change in slow wave frequency accompanying motility, and never a consistent change. Similar results were obtained in both human and dog small intestine.

In studies with microelectrodes, depolarizations which initiated action potentials inside a muscle cell were larger than depolarizations which did not (20). In studies using macroelectrodes there was not always a clear distinction during periods of motility between the amplitudes of slow waves associated with action potentials and those which were not. In these records, however, there was a clear distinction between average slow wave amplitudes during periods of motility and during periods of inactivity. This difference cannot be finally evaluated because intracellular recordings were rarely made during periods of inactivity and because extracellular recordings reflect the summation of depolarizing currents from many cells and are affected by the degree of synchrony as well as by voltages at individual cells. It seems likely, however, that the increased amplitudes recorded during motility with macroelectrodes arise, in part at least, from greater average depolarizations at cells in the field of the electrode and that this is also reflected in the occurrence of action potentials at some of these cells.

Propulsion seems to occur only under those conditions which favor the occurrence of spread of slow waves. This aboral spread of slow waves generally occurs only over short distances, from 10 to 40 cm at rates of 6–20 cm/second in the duodenum. If each slow wave spreads over the entire small intestine all segments should have the same frequency. This is not the case (1–11). Further, propulsion of a balloon in the duodenum does not affect slow wave frequencies in the jejunum or ileum and the spread of motility over the intestine after eating likewise does not alter slow wave frequencies in the ileum. In addition, waves of contraction spread over the intestine at a much lower rate than slow waves (23–25). Therefore, the presence or absence of motility is not directly determined by the spread of individual slow waves.

Action potentials are not conducted sufficient distances in the intestine to serve as a co-ordinating mechanism for motility (10, 12, 13). The slow waves appear to provide such a co-ordinating mechanism by ensuring that the intestinal muscle in a given area becomes excitable more or less simultaneously.

The depolarized (and presumably more excitable) area along the intestine can be calculated to be 20 cm long, assuming a conduction velocity of slow waves of 10 cm/second and a duration of the positive phase of the slow wave (corresponding to depolarization) of 2 seconds. Propulsive contraction rings in the dog intestine are only 1–2 cm wide. During balloon propulsion, inhibition of motility is present 1–2 cm ahead of the contraction ring. Some local factors therefore must cause changes in the magnitude of slow wave depolarizations over limited areas so that activation and inhibition are produced.

Acetylcholine and serotonin suggest themselves as local activating factors while epinephrine could function as an inhibitory one. Acetylcholine and serotonin increase slow wave amplitudes and cause the appearance of action potentials. Both are found in the small intestine and are released by distention (26, 27), which is the physiological stimulus to motility. Epinephrine, on the other hand, diminishes slow wave amplitudes and inhibits action potentials, though little is known concerning the mechanism of its release in the intestine.

Regulation of Slow Waves

As Ichikawa and Bozler (28) have pointed out, the fact that an external recording using a macroelectrode can be made from units the size of intestinal muscle cells indicates that adjacent cells are depolarizing relatively synchronously. Furthermore, slow waves often appear to spread along the intestine in a regular manner. Intestinal cells must therefore have some means of influencing one another. Conceivably this influence may be exerted by means of a chemical substance, by an electric current, or by mechanical strain.

Mechanical strain on adjacent cells induced by the contraction of neighboring cells in the longitudinal or circular muscle layer might provide a means whereby the influence of cells is transmitted. There is, however, no evidence that periodic contractions move down the resting intestine and slow wave frequency and conduction are not altered in the relaxed intestine after the administration of atropine or nicotine. Strain of longitudinal muscle cells by distention or by a contraction of circular muscle is probably involved in the mechanism whereby slow wave amplitude is increased locally, leading to action potentials and contraction. However, mechanical strain in itself does not seem adequate to account for the transmission of slow waves down the intestine.

Release of a chemical substance by nerves has been proposed as a mechanism controlling slow waves, but this study and others (2, 6) have shown that isolation of the intestine from its extrinsic nerves has little influence on size or spread of slow waves. Inhibition of ganglionic transmission by nicotine or hexamethonium similarly has no effect. Antagonism of acetylcholine by atropine diminishes the amplitude of slow waves but does not alter their conduction or frequencies. Others (9, 10) have shown that local anaesthetics like procaine alter the spread of slow waves but do not prevent their occurrence in the treated segment. The amounts of procaine required suggest that this effect may not be related to block of nerve conduction and indeed it has been suggested (10) that antagonism of the effects of acetylcholine is involved. Thus neither extrinsic nor intrinsic nerves seem to play a vital role in regulation of slow waves.

Some have considered the possibility that slow waves are transmitted electrically (6, 9, 10, 11). This would be conceivable if the intestinal muscle cells formed a syncytium. However, recent studies have provided no evidence of protoplasmic interconnections between intestinal cells (2, 3). Electrical transmission of slow waves between cells separated by high resistance membranes certainly cannot occur by the classical mechanism, local circuit spread. There is no evidence of a regenerative depolarization leading to an all-or-none response during each slow wave, and in fact there is some evidence that action potentials do not represent all-or-none responses of small intestinal muscle cells (20). When the amplitudes of slow waves are elevated during balloon propulsion there is no indication that the increased rate and extent of depolarization increases the velocity of conduction. In fact, the velocity of conduction tends to be diminished. These findings, too, are incompatible with classical concepts of electrical transmission (29). Electrical interactions of cells may possibly be explained in terms of an intrinsic instability of their limiting membrane, which tends to polarize and depolarize at a characteristic rate. Synchrony of this process in a large number of cells may be sufficient to act on nearby cells and to initiate depolarization in them. Accurate calculations of the expected conduction rates for such a process cannot be made since the required amount of depolarizing current or depolarization is unknown and the time required for the process is therefore also unknown. Assuming that the cells are 100 microns long and laid end-to-end, then only 5 milliseconds could be allowed from the beginning of depolarization of one cell and the attainment of sufficient depolarization to affect the next one if conduction is to occur at 10 cm/second. This seems insufficient time, and probably the cells are not excited end-to-end so that even less than 100 microns is covered with each cell-to-cell transmission. This would make the time available even less. Similar considerations apply to transmission by chemical diffusion. It is obvious that additional data will have to be considered before the nature of the process of cell-to-cell transmission is understood.

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(For EXPLANATION OF FIGURES and for FIGS. 1–7, see pp. 792–801.)

Explanation of Figures

Fig. 1. Dog anaesthetized with divinyl ether – chloralose. (1*a*) Relationship between balloon (3.5 cm long) in duodenum and electrode before propulsion. The proximal end of the balloon was 1 cm below the stab wound. (1*b*) Electrical activity during propulsion of a balloon past a duodenal electrode. The activity at the electrode before balloon inflation consisted of regular slow wave (1.5 mv amplitude and 20.4 per minute frequency without action potentials). A few seconds after inflation of the balloon, propulsion was initiated, but no change occurred in slow wave frequency. As the balloon moved to 3.0 cm so that it was under the recording electrode, small action potentials appeared. As the balloon moved to 3.5 cm below the wound so that its maximum circumference was past the electrode, the action potentials became larger and the slow wave amplitudes increased (to about 2 mv) but the frequency remained unchanged. As propulsion continued, action potential bursts continued to occur intermittently, but always on the positive (upward) phase of the slow wave. Slow wave amplitudes reached values of 2.5 to 3.5 mv as the balloon reached a position of 5 cm and was just distal to the electrode. Eventually, when the balloon had reached a position of 6.5 cm below the wound (1.5 cm below the electrode) action potentials and propulsion ceased temporarily and the slow waves were slightly diminished in amplitude to 2.0 to 2.5 mv. The area around the electrode was still excited and subsequently further propulsion and action potentials occurred. Movement of the balloon followed bursts of action potentials.

Fig. 2. Electrical activity of the dog intestine during serotonin infusions. Dog anaesthetized with pentobarbital sodium. Electrodes just distal to bile duct entrance (U.D.), in lower duodenum (L.D.), in jejunum (JEJ.) about 10 cm distal to ligament of Trietz and about 15 cm proximal to ileocecal junction (IL.). Bipolar records taken between upper and lower duodenum electrodes. Records in vertical array were not made simultaneously but were obtained within a 5-minute interval. A. Control records from the various electrodes are indicated. B. Records during the first 10 minutes of an intravenous infusion of serotonin (10 μg/kg/minute). Action potentials occurred intermittently at the duodenal electrodes, but not in the jejunum and ileum. Conduction, as measured in bipolar records, was not altered. Slow wave frequencies tended to be decreased at all electrodes and amplitudes were increased at duodenal electrodes. C. Control records after cessation of serotonin infusion. D. Records during the first 10 minutes of an intravenous infusion of 200 μg/kg/minute of serotonin. No changes were noted in any record except for a decrease in slow wave frequencies.

Fig. 3. Slow waves and action potentials after mechanical stimulation of the jejunum. Dog anaesthetized with pentobarbital sodium. Jejunum exposed at laparotomy and balloon inserted through a stab wound proximal to electrode. The jejunum was stroked gently but repeatedly with a blunt instrument at a point 0.5 cm distal to the recording electrode. As soon as a contraction ring was formed the stroking was stopped and recording begun. At the beginning of the record (arrow) the contraction ring was not pulsating. The slow waves were small and action potentials absent or small. Pulsation of the contraction ring began 9 seconds later as noted on the record. Thereafter, the slow waves became larger and action potential bursts appeared. They occurred in all phases of the slow wave. Bursts of action potentials occurred at a frequency which was independent of the slow waves and was determined by the pulsations of the contraction ring. Contractions which spread to the recording electrode were noted by a mark along the upper margin of the records.

Fig. 4. Inhibition of duodenal electrical activity by distention of a balloon. Dog anaesthetized with divinyl ether – chloralose. Balloon inserted through stab wound 1 cm distal to pylorus. Electrodes A, B, and C inserted 1, 4, and 6 cm below stab wound. A. The proximal edge of the balloon was just inside the stab wound when inflated and the maximum circumference of the inflated balloon was a few millimeters distal to the top electrode, A, when inflated with 5 ml air. Before inflation of the balloon, slow waves of 2.2 to 3.5 mv occurred with a frequency of 18.1 per minute and were accompanied by small (about 0.4 mv) action potentials. During and after inflation of the balloon action potentials disappeared and the slow waves diminished in size to about 1.4 mv. The slow wave frequency remained 18.1 per minute. After about 30 seconds larger (1 mv) action potentials returned and persisted and the slow waves varied between 2.2 and 3.0 mv with no alteration in frequency (not shown). B. When recording was done from the middle electrode, B, during an identical experiment, action potentials were inhibited by balloon distention, but did not return for several minutes until the balloon was deflated. Slow wave frequency increased from 17.1 to 17.6 per minute just after balloon inflation, but this increase in frequency was not maintained and may have been coincidental. Slow waves were of an unusual configuration. The amplitude was not clearly altered during inhibition of action potentials, but varied about 1 mv throughout the procedure. C. When recording was done from the lower electrode C, results similar to those described at B were obtained. The action potentials were inhibited until the balloon was deflated. Slow wave frequencies increased from 17.6 to 17.9–18.2 per minute while the balloon was inflated, but this may have been coincidental. Slow wave amplitudes were reduced from between 1.0 to 1.6 mv to between 0.4 to 1.1 mv during inhibition of action potentials.

FIG. 5. Bipolar recording of electrical activity of dog duodenum during balloon propulsion. Dog anaesthetized with divinyl ether – chloralose. Electrodes 2.5 cm apart and arranged so that an upward deflection occurred when the upper electrode became positive with respect to the lower one. Proximal end of the balloon just distal to upper electrode when 0 cm was noted on record. Before balloon inflation, occasional small action potentials occurred. The interval between the bipolar peaks was 200 to 280 milliseconds indicating a conduction velocity of 8.9 to 12.5 cm/second. During inflation of the balloon, bursts of action potentials occurred and the balloon was propelled onward 2.5 cm so that its proximal end was just beneath the upper electrode and its maximal circumference was between the two electrodes. The conduction velocity decreased to about 5 cm/second and action potentials became small, infrequent, and seemed to occur only at the upper electrode. The balloon remained stationary for several seconds as the conduction velocity gradually recovered to 7.8 to 8.9 cm/second. Then propulsion began again, accompanied by bursts of action potentials at both electrodes. By the time the balloon had been moved 4.0 cm and was just past the lower electrode, the conduction velocity had returned to control values (8.9 to 13.2 cm/second). It remained at those velocities during the rest of the record as the balloon was propelled to an ultimate distance of 7.2 cm. As usual during propulsion, action potentials were in phase with slow waves and slow wave frequencies remained essentially unchanged throughout the record. Bipolar records represent the algebraic summation of events at two electrodes and amplitudes of bipolar slow waves are therefore no indication of the amplitudes of the individual monopolar records.

FIG. 6. Effect of eating on electrical activity of the human ileum. A. Control record of electrical activity before eating showing two slow waves with amplitudes of 0.5 to 0.6 mv. B. Record taken soon after the ileum began to show activity. Slow waves were larger, about 1 mv, and small action potentials were appearing in phase with slow waves. C. Record after end of meal. The ileum had become extremely active and evacuations occurred repeatedly. Larger action potentials accompanied the contractions which spread to the electrode. They were in phase with the slow waves, which had reached amplitudes of 1.0 to 1.5 mv. D. Record during contractions with repeated evacuations. Action potentials were large and occurred throughout the positive phase of the slow waves. Slow wave amplitudes were from 1.5 to 2.0 mv. Slow wave frequencies were not altered significantly in any of these records.

FIG. 7. Effect of various drugs on electrical activity of the human ileum. A. Control record showing slow waves (0.4 to 0.6 mv) without action potentials. B. Slow waves (2 to 2.5 mv) containing action potentials in phase during intermittent contractions induced by the intravenous administration of 2 mg of serotonin. C. Slow waves (1.2 to 1.4 mv) containing action potentials in phase. These corresponded with the intermittent contractions superimposed on the spasm induced by the intravenous administration of morphine sulphate (16 mg). D. Inhibition of morphine-induced electrical activity (as well as mechanical activity) following the intravenous administration of 5 mg of nalorphine HCl. The slow waves were reduced in amplitude to less than 1.0 mv although the initial slow wave appeared larger owing to a shift of baseline upward concomitant with the positive shift of the slow wave.

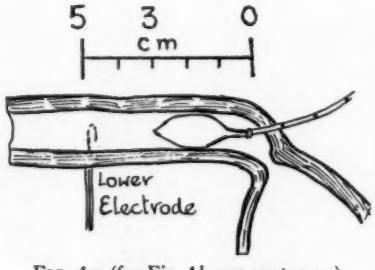

FIG. 1*a* (for Fig. 1*b*, see next page).

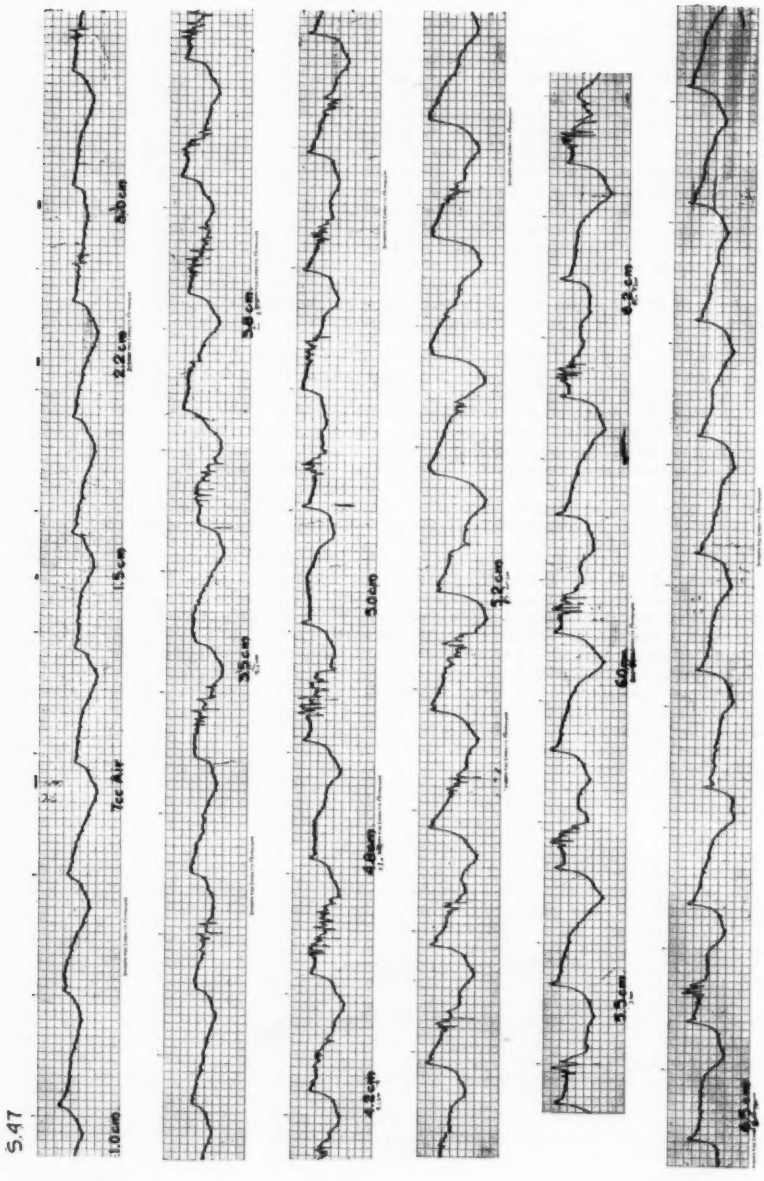

FIG. 1b.

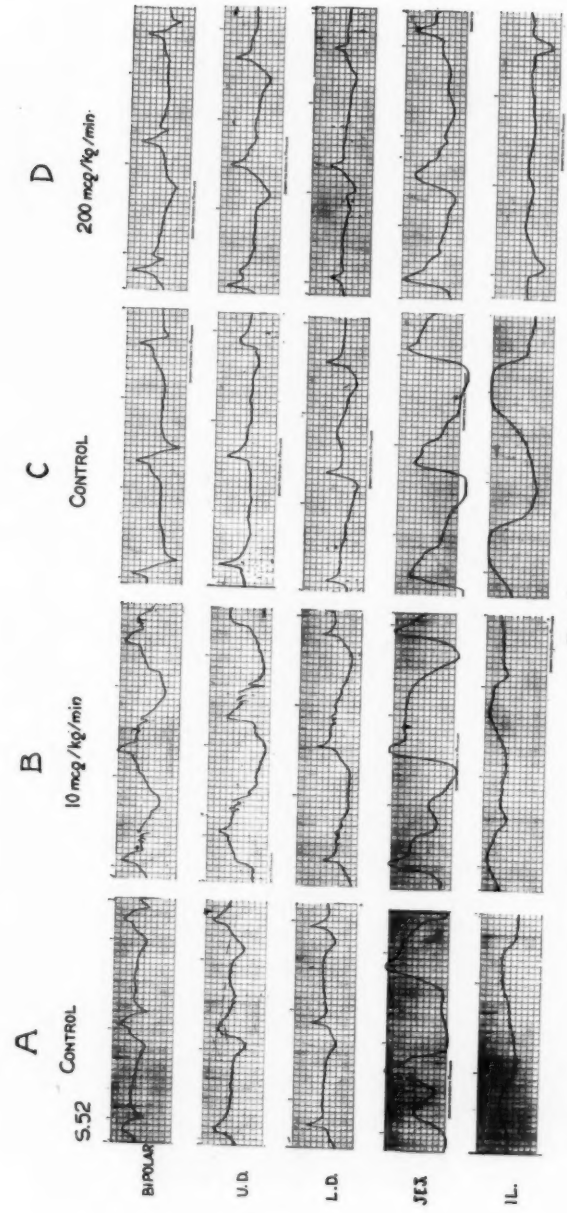
A
CONTROL
S.52
B
10 mcg/kg/min
C
CONTROL
D
200 mcg/kg/min
BIPOLAR
U.D.
L.D.
JEJ.
IL.

FIG. 2.

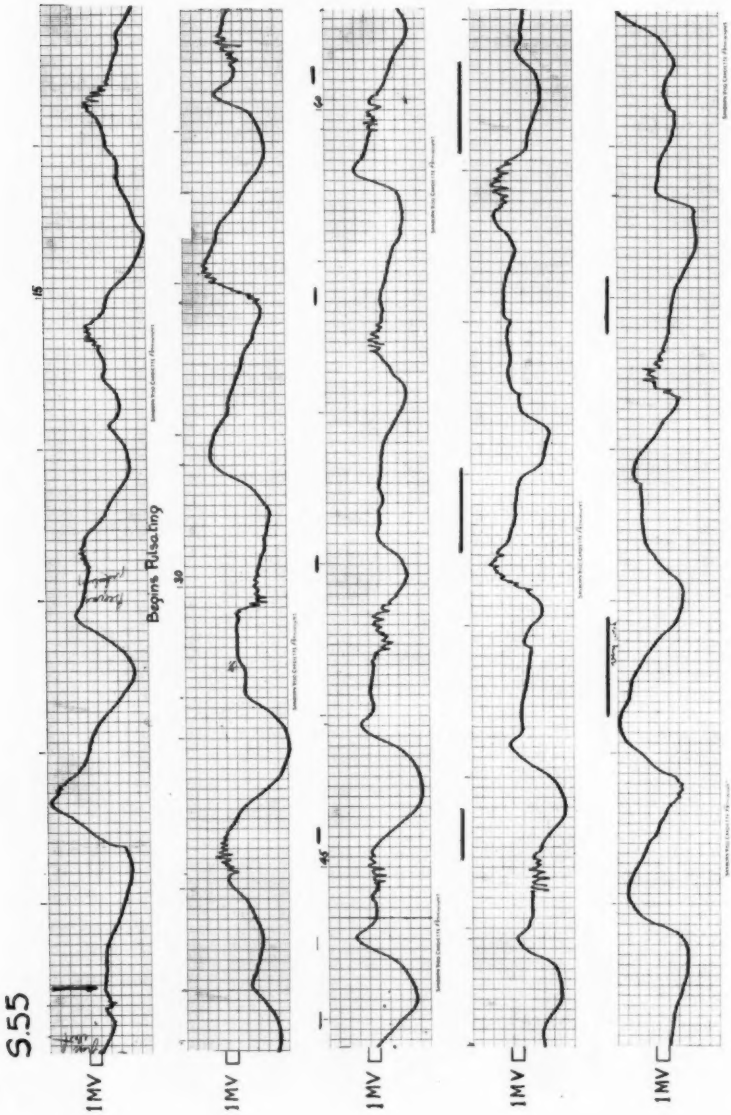

FIG. 3.

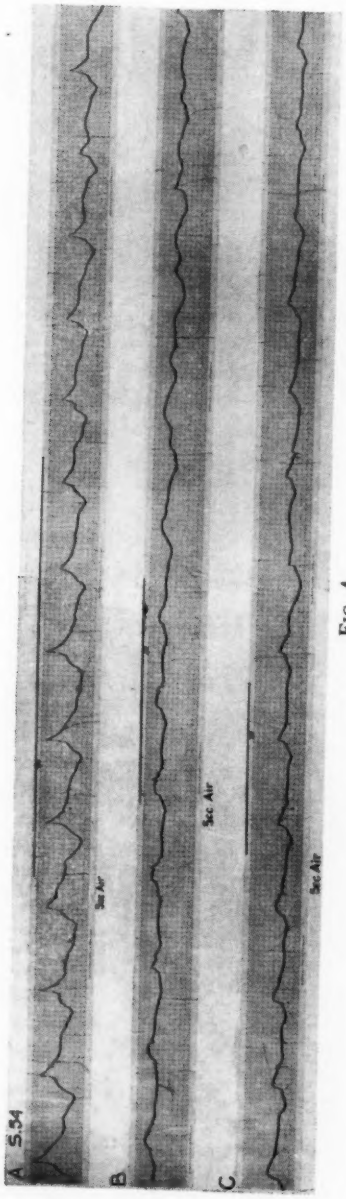

FIG. 4.

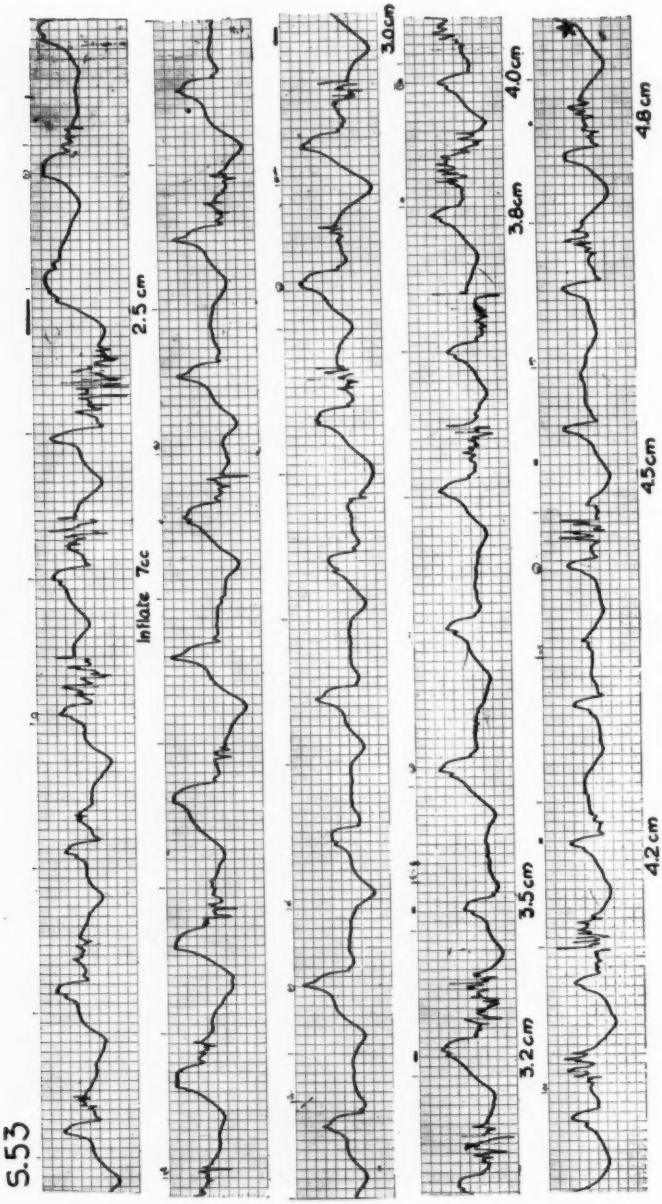

FIG. 5.

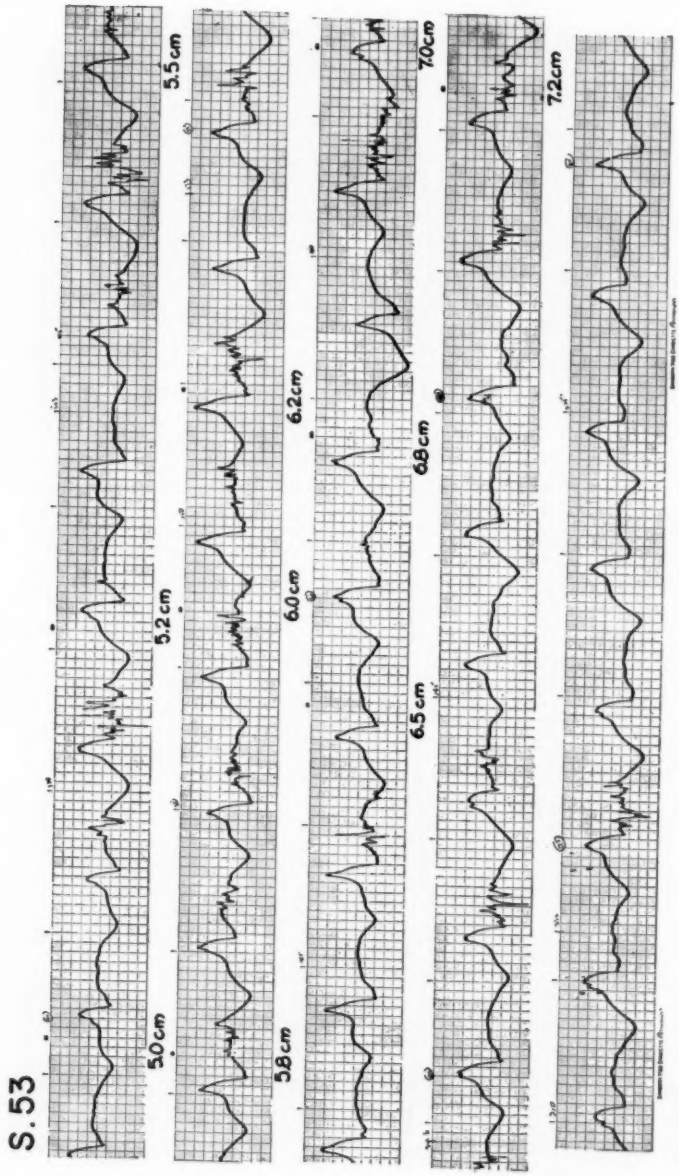

FIG. 5 (*concluded*).

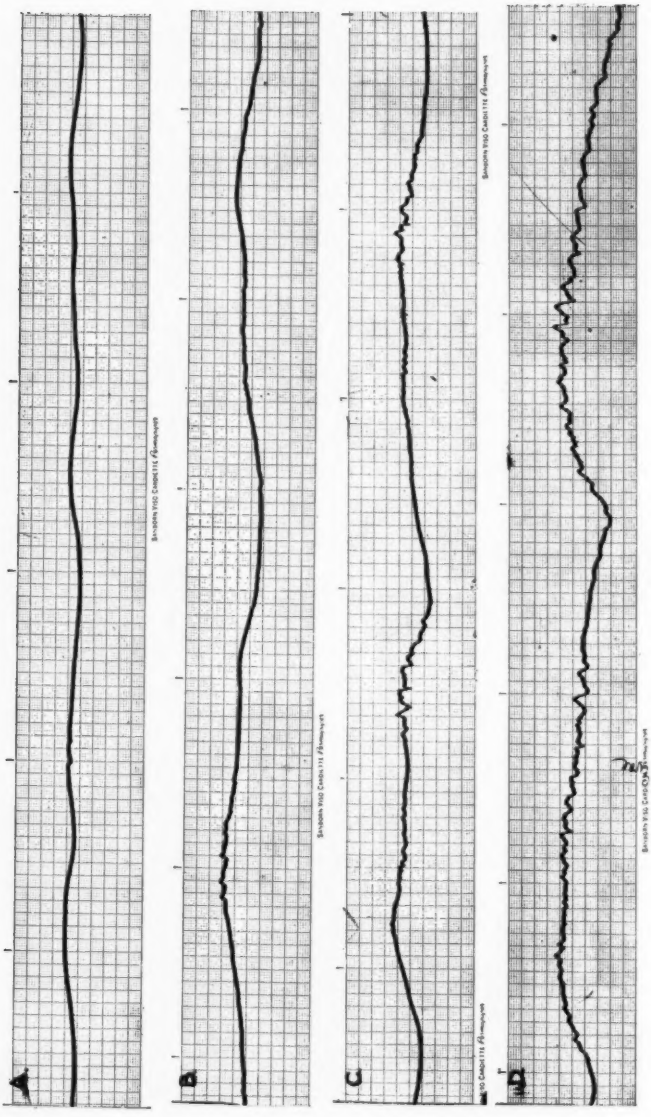

FIG. 6.

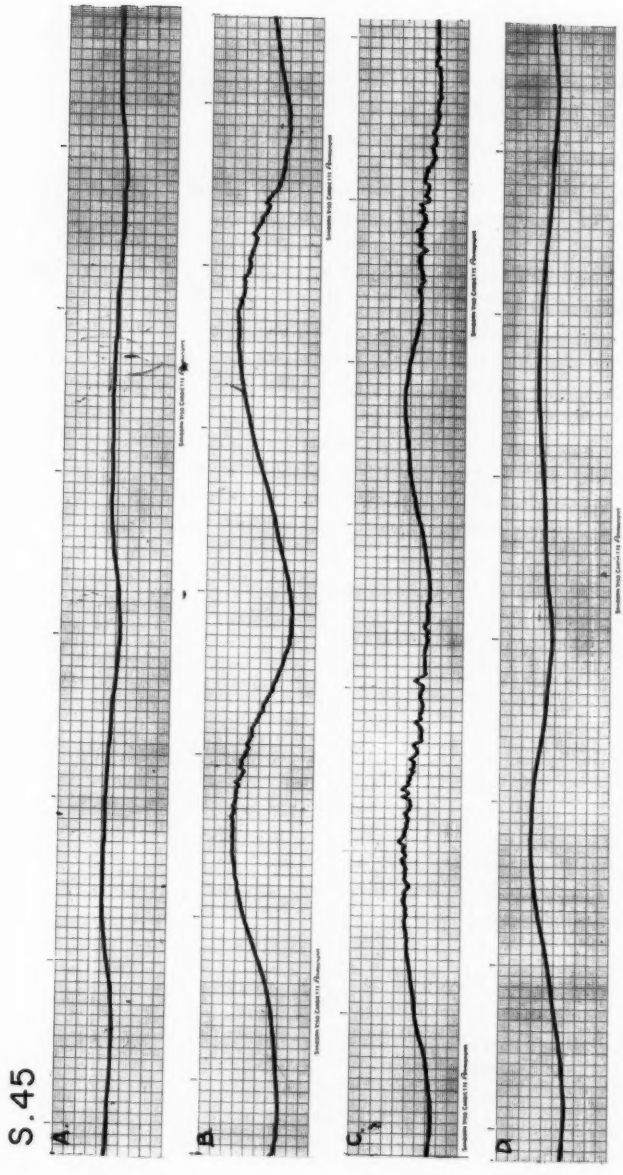

FIG. 7.

NOTES

THE LACK OF EFFECT OF ASCORBIC ACID ON THE THYMOLYTIC ACTIVITY OF HYDROCORTISONE

N. R. STEPHENSON

Bacchus and co-workers reported that ascorbic acid enhanced cortisone-induced gluconeogenesis in adrenalectomized mice (1) and diminished the excretion of urinary 17-ketosteroids following the administration of cortisone to adrenalectomized rats (2, 3). In addition, treatment with ascorbic acid apparently maintained the circulating levels of 17-hydroxycorticosteroids for 72 hours after the injection of cortisone in adrenalectomized rats (4). In vitro studies by Bacchus (5, 6) suggested that liver tissue of rats treated with ascorbic acid metabolized cortisone to a lesser extent than did liver tissue from untreated control animals.

These observations suggest that ascorbic acid might increase the thymolytic activity of hydrocortisone by maintaining a higher than normal concentration of the steroid in the circulating plasma following its subcutaneous injection. The results of experiments designed to investigate this possibility are reported in this communication.

Methods

Weanling rats derived from an inbred Wistar strain were employed as the test animals. The ascorbic acid was dissolved in isotonic saline and neutralized with 0.1 *N* NaOH just before it was injected into the rats. The hydrocortisone was administered either in the aqueous medium containing the neutralized ascorbic acid and 10% ethyl alcohol, or in corn oil. The injections were made over a period of 2 or 3 days. In one of the experiments, an intraperitoneal injection of ascorbic acid was given to the rats the night before the day of the test. Approximately 18 hours after the last injection of either hydrocortisone, ascorbic acid, or both, the thymus gland was removed carefully, blotted on filter paper, and weighed on a Roller-Smith torsion balance. The response to the treatment was estimated by dividing the wet weight in milligrams of the thymus gland by the final body weight in grams.

Results

In the first experiment the neutralized ascorbic acid was injected subcutaneously with and without hydrocortisone into the weanling rats at dose levels of 10 mg per 100 g body weight either as the total dose, or as the dose per injection. The data in Table I clearly indicate that the concomitant administration of ascorbic acid had no effect on hydrocortisone-induced thymic involution in weanling rats.

In the second experiment, the neutralized ascorbic acid was administered by intraperitoneal injection and the first of the seven doses (10 mg per 100 g body

TABLE I

Influence of ascorbic acid on the thymolytic activity of hydrocortisone when injected subcutaneously in the same medium

Treatment: 2 injections per day for 3 days (total dose)	Av. relative thymus wt. ± S.E.		
	Without hydrocortisone, mg/100 g of rat	With 0.3 mg hydrocortisone (total dose), mg/100 g of rat	% reduction in thymus wt. due to hydrocortisone
Saline + 10% ethyl alcohol	432 ± 9.2 (19)‡	284.4±8.6 (19)	34.3%
4.2 mg ascorbic acid*	413.6±16.4 (10)	279.4±8.3 (10)	32.5%
26 mg ascorbic acid†	426.9±18.7 (10)	282.4±8.9 (10)	33.8%

*Total dose of neutralized ascorbic acid = 10 mg per 100 g of rat.
†Each injection of neutralized ascorbic acid = 10 mg per 100 g of rat.
‡Number of rats per dose group in parenthesis.

weight) was given approximately 18 hours before the hydrocortisone was injected subcutaneously. Thereafter the neutralized ascorbic acid was injected intraperitoneally thrice daily for 2 days at the same time as the hydrocortisone in corn oil was administered subcutaneously. The results shown in Table II demonstrate that pretreatment with ascorbic acid followed by concomitant injections with hydrocortisone did not significantly influence the thymolytic activity of the corticosteroid.

TABLE II

Effect of pretreatment of rats with ascorbic acid on hydrocortisone-induced thymic involution

Treatment (3 injections per day for 2 days (total dose))	Av. relative thymus wt. ± S.E.		
	Without hydrocortisone, mg/100 g of rat	With 0.2 mg hydrocortisone (total dose), mg/100 g of rat	% reduction in thymus wt. due to hydrocortisone
Corn oil (subcutaneous)	410.3±12.1 (10)†	240.9±16.4 (10)	41.3%
Corn oil (subcutaneous) + 32 mg ascorbic acid* in isotonic saline (intraperitoneal)	402.8±16.0 (11)	248.5±14.5 (11)	38.3%

*An initial intraperitoneal injection of the neutralized ascorbic acid in saline was given approximately 18 hours before the corn oil injections were started. Each injection of neutralized ascorbic acid was equivalent to 10 mg/100 g of rat.
†Number of rats per dose groups in parenthesis.

It is apparent from this investigation that amounts of ascorbic acid which Bacchus and co-workers (1) used to enhance the cortisone-induced gluconeogenesis in the liver of the adrenalectomized mouse did not influence in any way either the thymus gland or the involution brought about by hydrocortisone in the weanling rat. It is unlikely, therefore, that the concentration of exogenous hydrocortisone was maintained at an elevated level for a longer period of time in the blood of the rats treated with ascorbic acid than it was in the blood of the untreated animals.

Acknowledgment

The hydrocortisone employed in this investigation was kindly supplied by Merck and Co. Ltd. The author wishes to thank Mr. P. J. Kavanagh for his valuable technical assistance.

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AMINO ACID – PROTEIN INTERACTIONS[1]

C. GODIN

When individual amino acids are incubated in simple amino acid – protein systems, binding of amino acids to the protein molecule results (1, 2, 3, 4). In this note we would like to report some evidence showing that the free amino groups and the free carboxyl groups of a protein molecule are partly responsible for this binding.

Crystalline zinc insulin, acetylated insulin (5), and esterified insulin (6) were incubated with DL-phenylalanine-2-C^{14} at 37° C and pH 7.4. After 2 hours, the incubation mixtures were dialyzed extensively and the proteins precipitated with trichloroacetic acid. The radioactive proteins were treated with fluorodinitrobenzene (FDNB) and the resulting insoluble dinitrophenyl-proteins (DNP-proteins) isolated. The C^{14}-phenylalanine liberated during reaction with FDNB was isolated as the DNP-derivative by extracting the acidified reaction mixture with ether (fraction A, Table II). After complete acid hydrolysis of the DNP-proteins, N-terminal phenylalanine was isolated by paper chromatography (fraction B, Table II).

TABLE I

Binding of DL-phenylalanine-2-C^{14}*

Proteins	c/min/mg proteins	μmoles phenylalanine bound/g proteins	c/min/mg DNP-proteins	% phenylalanine lost during dinitrophenylation†
Insulin	277	4.9	206	6.9
Acetylated insulin	243	4.3	180	7.3
Esterified insulin	794	14.1	624	1.8

*Incubation mixture: DL-phenylalanine-2-C^{14}, 2.6 mg (16 micromoles), 3.44×10^8 c/min/mg. Proteins, 10 mg. Phosphate buffer 0.1 *M*, pH 7.4, 2 ml. Incubation time 2 hours at 37° C. The results were corrected for self-absorption.
†In order to calculate the loss, it was assumed that 1 mg of DNP-insulin corresponds to 0.8 mg insulin.

The phenylalanine present in the hydrolyzate was also transformed into the DNP-derivative and isolated (fraction C, Table II). The results obtained are given in Tables I and II. The results show a small decrease in specific activity after dinitrophenylation. Esterified insulin binds much more C^{14}-phenylalanine

[1]This work was supported by a grant from the National Research Council of Canada.

TABLE II

Distribution of DL-phenylalanine-2-C^{14}*

Proteins	c/min/mg DNP-phenylalanine		
	Fraction A	Fraction B	Fraction C
Insulin	35	121	52
Acetylated insulin	35	18	55
Esterified insulin	20	470	5

*For meaning of A, B, and C, please refer to text.

than unesterified and acetylated insulin even if it is partly insoluble in the incubation mixture. It is possible that the esterified protein is partly denatured and that the remaining active groups are more available for binding. Table II shows a decrease in specific activity of fraction B of acetylated insulin and of fraction C of esterified insulin. Fraction B represents C^{14}-phenylalanine bound to the protein molecule but which is still able to react with FDNB. The amino acid is probably bound to the protein by its carboxyl group. In acetylated insulin, the amino groups are not free and we obtain a decrease in binding of phenylalanine in fraction B. It can be assumed then that normally there is interaction between the carboxyl group of the amino acid and the free amino groups of the protein molecule. The reverse situation is found in the case of fraction C and esterified insulin and therefore we can assume interaction between the amino group of phenylalanine and the free carboxyl groups of the protein molecule.

We failed to isolate any small addition products from partial acid hydrolyzates of the radioactive proteins. Trypsin is known to liberate alanine from the C-terminal end of insulin (7). Trypsin did not liberate any alanyl-C^{14}-phenylalanine from radioactive insulin. Carboxypeptidase failed to liberate any C^{14}-phenylalanine from radioactive insulin. This indicates that binding at 37° C probably does not involve the formation of peptide bonds.

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